FEBRUARY 10, 1943

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THE JOURNAL OF NUTRITION

VOLUME 25 NUMBER 2

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Official Organ of the American Institute of Nutrition
PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.
Price, $5.00 per volume, Domestic; $5.50 per volume, Foreign

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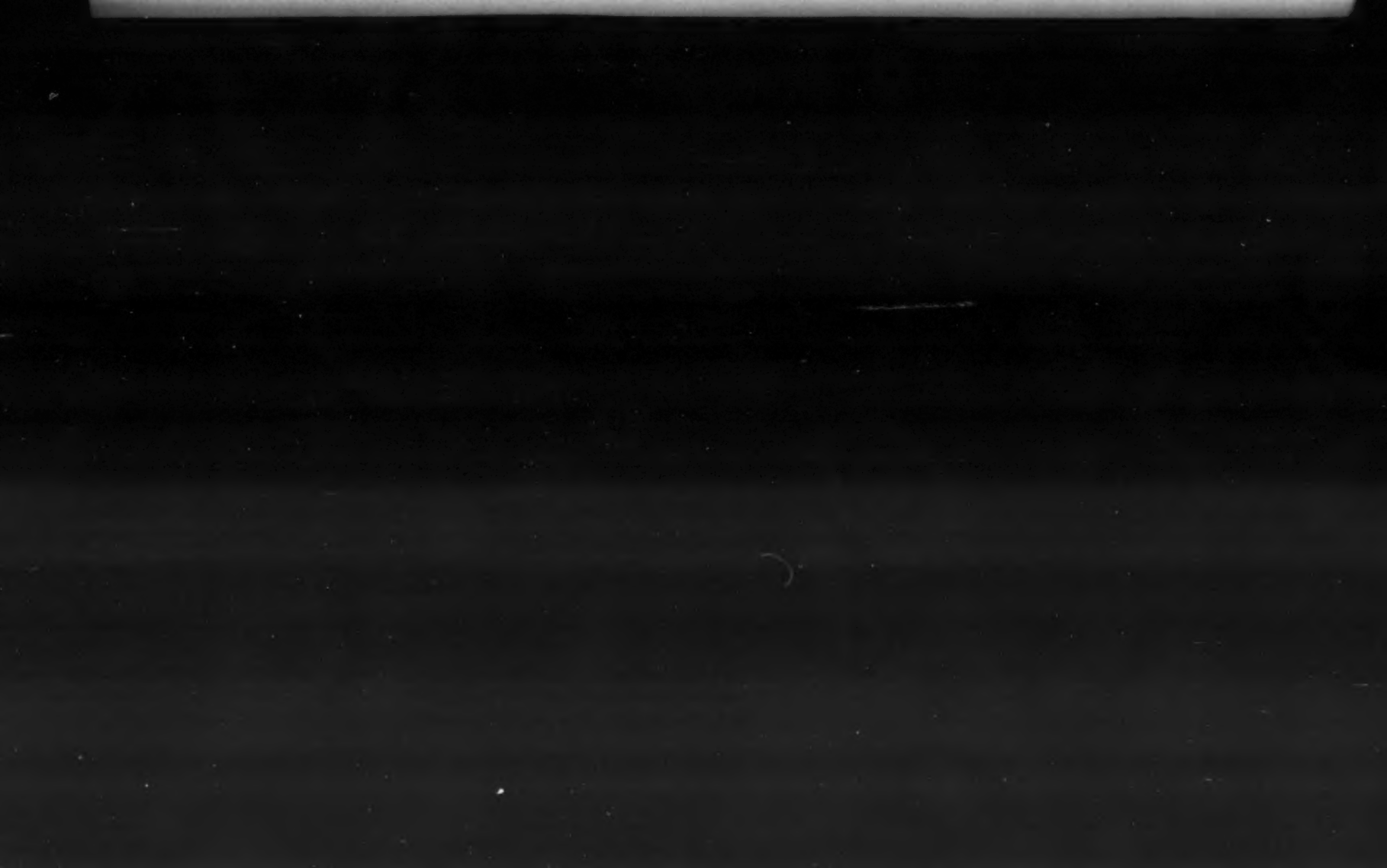

STUDIES ON CAROTENOID METABOLISM

III. THE EFFECT OF A HIGH VITAMIN A DIET ON THE CAROTENOID METABOLISM OF CHICKENS [1]

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(Received for publication July 20, 1942)

In an earlier communication from this laboratory (Deuel, Halliday, Hallman, Johnston and Miller, '41) it was demonstrated that the feeding of vitamin A in large doses in the form of shark liver oil to Guernsey cows was followed by a pronounced lowering in the carotene level of the milk, and at the higher levels of administration of the vitamin a marked augmentation in the vitamin A content of butterfat was noted. Later it was found (Deuel, Hallman, Johnston and Mattson, '42) that the depression in milk carotene was a resultant of the feeding of vitamin A itself rather than of other components in the shark liver oil, inasmuch as it also occurred after the administration of a vitamin A concentrate. Moreover, it was noted that a similar response could be elicited in Holstein cows where the normal level of milk carotene is approximately 50% less than that of the Guernsey; also evidence was given which indicated that the intensity of the effect of vitamin A was proportional to the dosage through a certain range. Because a similar variation in blood carotene was found, it was

[1] Papers I and II of this series were not numbered. These are referred to in the Literature Cited, respectively, as Deuel et al. ('41, '42).

The data reported in this paper were presented before the meeting of the Southern California Section of the Society of Experimental Biology and Medicine on May 21, 1942. They are offered by Fred H. Mattson in partial fulfillment of the requirements for the degree of Master of Science.

THE JOURNAL OF NUTRITION, VOL. 25, NO. 2
FEBRUARY, 1943
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suggested that these phenomena were best explained on the basis of the development of a new enzyme system capable of destroying carotene. It was postulated that the formation of this enzyme system is stimulated by a high concentration of vitamin A in the tissues. The slow rate of development of the maximum decrease in carotene (4 to 6 weeks after the initiation of vitamin A feeding) as well as the tardy return to the normal value after cessation of such supplements (8 to 10 weeks) would seem to support such a theory. It is also possible that an increased activity of an enzyme system already present may be brought about by the high vitamin A intake.

In the present work the chicken has been found to be a satisfactory animal for a further investigation of this problem. Carotene retentions and depositions in the rat and pigeon even after a high carotenoid diet were found to be too small to be useful for this study. The levels of blood and liver carotene and carotenols (chiefly xanthophyll and zeaxanthin) have been compared in birds receiving a normal and high intake of vitamin A. Also a study was made of the absorption of these pigments from the gastrointestinal tract in the two groups of animals so that it might be determined whether absorption is a factor in the depressed carotenoid metabolism accompanying a high vitamin A intake.

EXPERIMENTAL

Two series of tests were made. The first was carried out on eight white Leghorn chickens [2] which had received for the previous 6 weeks the A.O.A.C. vitamin D assay diet supplemented with 50 units per gram of synthetic vitamin D_3.[3] The corn in one diet (diet I) was entirely yellow, while in diet II, three-fourths of it was replaced with white corn. Two of the birds which had been on each diet were immediately sacrificed to determine whether a different level of liver carotenoid ob-

[2] These chickens were kindly furnished by the Emeryville laboratories of the California Packing Corporation.

[3] Dupont.

tained. Two chickens previously on diet I were continued for 2 weeks on a very high carotenoid diet (diet IV) to ascertain whether additional carotene and carotenols could be stored. Finally, two birds previously fed diet II were continued for 2 weeks on a practically carotenoid-free diet (diet III), and then for a like period on a carotenoid-rich diet, diet IV. The chickens were then sacrificed and the carotene and carotenols determined in the liver. One chicken (no. 8) received in addition 9300 I.U. of vitamin A daily during these last two periods. Diets III and IV had the composition shown in table 1.

TABLE 1

The per cent composition of the carotenoid-low diet (III) and a high carotenoid diet (IV).

COMPONENT	DIET III	DIET IV
Whole wheat flour	50	25
Skimmed milk powder	30	30
Alfalfa		25
Yeast [1]	10	10
NaCl	2	2
$CaCO_3$	2	2
Coconut oil	5.8	5.8
Super biotol [2]	0.2	0.2

[1] Strain K, Anheuser Busch.

[2] Contained 3000 I.U. of vitamin A and 400 A.O.A.C. units of vitamin D per gram.

In the second series of tests forty-two 2-week-old white Leghorn chickens were used. They were divided into two groups. Each chicken in group II received daily throughout the test a supplement of 0.1 cc. of shark liver oil which contained 9300 I.U. of vitamin A,[4] while the remaining animals were used as controls (group I). After 2 weeks on the basal carotenoid-low diet (diet III), some of each group were killed; the rest were then placed on the high carotenoid diet (diet IV) for an additional 2 weeks, after which they were sacrificed.

[4] The oil was furnished by California Packing Corporation and contained 93,000 I.U. per cubic centimeter.

The livers were extracted for analysis by the Petersen, Hughes and Freeman modification ('37) of the Guilbert ('34) method. Carotene was determined by the method of Koehn and Sherman ('40), using a Klett-Summerson photoelectric colorimeter, while a similar colorimetric procedure with the same filter was employed for the carotenols, using a conversion factor of 0.932 which was found by Kuhn and Brockmann ('32) to be identical for xanthophyll (lutein) and zeaxanthin. Vitamin A determinations were carried out on the saponified material with the Beckman spectrophotometer, using petroleum ether as a solvent. Blood carotene was determined by a modification of the method of Davis and Madsen ('41) whereby the carotenols are separated from the petroleum ether solution of the carotenoids by extraction with 85% ethyl alcohol. The carotenols were determined with the photoelectric colorimeter as above. The minimum amount of carotene or carotenols which could be determined was 0.16 μg. per gram for a 5-gm. liver and 0.08 μg. per gram for a 10-gm. liver; with the quantities of blood available, concentrations below 8 μg. per 100 cc. could not be estimated. In the succeeding tables, values lower than these are considered zero.

In the tests on the absorption of the carotenoids, five birds each from groups I and II (second series) were placed in special metabolism cages and the food intake (diet IV) was weighed. The stools were collected each 24 hours and preserved under 95% alcohol in the dark until the end of the test. The total mass of alcohol and feces was saponified and the carotenoids extracted with petroleum ether. The saponification and extraction were repeated a second time. The carotene and carotenols were then determined on an aliquot part of the extract.

RESULTS

A summary of the results on the first series is given in table 2; the data on the second series are summarized in table 3, while the results of the absorption tests are found in table 4.

DISCUSSION

As in the earlier tests with cows, the carotenoid pigments are markedly lowered in the blood when large doses of vitamin A are given. In the present tests on chickens it was also shown that a similar condition obtains in the liver. In contrast to the cows where carotene is the main pigment, in the chicken the chief carotenoids are the carotenols. Although there is not always a decrease in the very low level of carotene in the liver, the decrease in carotenols is extremely marked. Thus in series I in single experiments, the carotenol level of 7.5 and 16.7 μg. was noted in animals with and without the high vitamin A supplement, respectively; in series II the variations were somewhat more marked, the average values being 4.3 and 19.5 μg., respectively.

Also there is a concomitant decrease in blood carotene and carotenol. Whereas the normal values for these components

TABLE 2

The content of carotenoids and vitamin A in the livers of chickens previously on various carotenoid intakes.

CHICKEN NUMBER	PRELIMINARY DIET	BODY WEIGHT		LIVER WEIGHT	CONTENT PER GRAM OF LIVER		
		When received	When killed		Carotene	Carotenols	Vitamin A
		gm.	*gm.*	*gm.*	*μg.*	*μg.*	
Killed immediately							
1	II	270	270	8.71		0	
2	II	250	250	6.73		0	
3	I	410	410	9.76		3.85 [1]	
4	I	500	500	10.29		4.62 [1]	
After 2 weeks on diet IV							
5	I	525	485	11.98	2.25	15.7	
6	I	450	430	11.30	1.35	15.0	
After 2 weeks on diet III followed by 2 weeks on diet IV							
7	II	420	575	21.26	2.32	16.7	90
8 [2]	II	300	475	15.90	1.32	7.55	1800

[1] Total carotene plus carotenols.

[2] Received also 0.1 cc. shark liver oil daily containing 9300 I.U. of vitamin A.

TABLE 3

The average carotene, carotenol and vitamin A contents in the livers and blood of chickens.

	GROUP I (NO SUPPLEMENT)		GROUP II (9300 I.U. VITAMIN A DAILY)	
	Diet III for 2 wk.	Diet III for 2 wk. Diet IV for 2 wk.	Diet III for 2 wk.	Diet III for 2 wk. Diet IV for 2 wk.
Number of tests	5	16	6	15
Average body weight in grams [1]				
Start (2 weeks)	91	84	76	97
4 weeks	137	147	128	169
6 weeks		221		224
Number of deaths	0	0	1	5
Liver				
Weight in grams	5.20	7.20	4.93	7.67
Carotene, micrograms per gram [2]	0.16 ± 0.07	0.69 ± 0.12	0.62 ± 0.19 **2.30**	0.68 ± 0.15
Carotenols, micrograms per gram [2]	0.32 ± 0.09	19.5 ± 2.8	3.95 ± 0.68 **5.25**	4.35 ±1.1 **5.05**
Vitamin A, International Units per gram [2]	7.9 ± 0.9	117.0 ± 7.4	5327 ± 630 **9.09**	4660 ± 1025 **4.42**
Blood plasma				
Number of samples [3]	1	3	1	2
Carotene, micrograms per 100 cc.	0	11.7	0	0
Carotenols, micrograms per 100 cc.	0	790	60	0
Vitamin A, International Units per 100 cc.		850		806

[1] The averages include only the weights of those chickens which survived.

[2] Including the standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations. The figures in bold face type are the quotients of the mean difference and the standard error of the mean difference, when each series in group II is compared with the corresponding series in group I. When this exceeds 3.00, the differences are considered statistically significant.

[3] Samples were obtained from all chickens. This gives the number of pooled samples analyzed separately.

TABLE 4

The comparative absorption of carotene and carotenols in chickens receiving diet IV with (group II) or without (group I) vitamin A supplement. Five chickens in each group.

GROUP	AVERAGE FOOD EATEN	AVERAGE CAROTENE IN MICROGRAMS FOR THE WEEK			AVERAGE CAROTENOLS IN MICROGRAMS FOR THE WEEK		
		Eaten	In feces	Absorbed	Eaten	In feces	Absorbed
	gm.						
I	136	4062	1104	2958	9002	5400	3602
II	138	4142	1024	3118	9174	6120	3054

were found to be 11 and 790 μg. per 100 cc. of plasma, respectively, no determinable amount was present in the blood plasma of the birds on the high vitamin A supplement. The decline in blood carotenoids is greater than has been noted in any tests on cows where as much as 4,200,000 I.U. of vitamin A were given over a longer period.

It may appear that the results on carotenoids in the two series of tests in table 3 are conflicting. In the chickens which received only the carotenoid-low diet, the liver and blood carotenoids were somewhat higher in the vitamin A-supplemented group than in the control group. On the other hand, the opposite was found in chickens which received the carotenoid-rich diet for an additional 2 weeks. However, the liver and blood carotenoids in chickens of groups I and II which received only diet III are minimum values as is also true of the values found in group II after the chickens received diet IV. Sharply contrasted with these values are the very much higher levels found in animals on a normal vitamin A intake with a carotenoid-rich diet. Thus, in the unsupplemented group (group I), liver carotene is increased from 0.16 to 0.64 μg. per gram after 2 weeks on the carotenoid-rich diet, while liver carotenols are augmented from 0.32 to 19.5 μg. per gram. Likewise blood carotenols change from zero to 790 μg. per 100 cc. In the vitamin A-supplemented group (group II), no similar difference follows the administration of the carotenoid-rich diet. Liver carotene is unchanged (0.62 and 0.68 μg. per gram) while the liver carotenols are at a similar low value in both series (3.95

and 4.35 μg. per gram, respectively). Likewise, although a measurable amount of blood carotenols was present in the first case (60 μg. per cent), no trace of carotene or carotenols was found in the blood after the carotenoid-rich diet had been given for 2 weeks.

According to our earlier suggestion, the decrease in carotene in the blood and butterfat of cows receiving large supplements of vitamin A is probably caused by the development of a non-specific enzyme system capable of destroying not only vitamin A, but carotene. If one accepts a similar explanation here, then it is evident that the enzyme is still less specific and that it can also destroy the carotenols. The carotenol, xanthophyll, is not considered capable of transformation into vitamin A in the chicken (Kline, Schultze and Hart, '32).

The level of carotenoid pigments in the liver and blood of chickens is also markedly influenced by the level of these components in the diet. When chickens were kept on diet II (series I) or diet III (series II), both of which were low in carotenoids, the carotene and carotenols reached such low levels that none or only small quantities could be detected in the liver or blood. Thus, the liver carotene varied between 0.0 and 0.62 μg. per gram, while blood carotene was so low it could not be determined; the liver carotenols varied between 0 and 3.95 while the maximum blood carotenols amounted to 60 μg. per 100 cc.

On the other hand, a marked rise both in carotene and carotenols occurred in the chickens receiving a high carotenoid intake (diet I or IV). Liver carotenols as high as 19.5 μg. per gram liver obtained in group I after 2 weeks on diet IV, while the maximum carotene level was 2.32 μg. per gram liver. Blood carotenols were 790 μg. per 100 cc. blood plasma in group I, while the carotene amounted to 12 μg. per cent. This value for blood carotenoids compares with the levels obtained with Guernsey and Holstein cows on green feed, although in these cases the pigment is almost exclusively carotene.

In all cases where the carotenoid pigments were lowered either by the administration of a carotenoid-low diet or by

the simultaneous administration of large doses of vitamin A along with a diet containing a high carotenoid content, a marked alteration in the normal yellow pigment in the combs, wattles, shanks, and eye sockets was very apparent.

The lower content of carotenoids in the birds receiving the high vitamin A supplements cannot be explained as due to failure to absorb these components. The average carotene absorbed during a 7-day period was 3118 μg. in the vitamin A supplement group (group II) and 2958 μg. in the control group (group I); the mean carotenol absorption was 3602 and 3054 μg. in groups I and II, respectively. If the application of these experiments can be extended to cows, the altered carotene in the milk and blood is also not to be attributed to an upset in absorption by the vitamin A supplemented animals.

The present experiments also prove the marked ability of the chicken to destroy or change beta carotene. Although it has been known for a long period that the carotenols are the chief pigments in this species, the present tests indicate that the absence of carotene from the tissues is not due to the failure in absorption.

The continued administration of the vitamin A supplement markedly increased the vitamin A stores in the liver. Thus the average value for the control group (I) in series II was 117 I.U. per gram of liver, while that of the vitamin A-supplement group (II) was 4660 I.U. per gram. However, no significant difference in the level of vitamin A in the blood could be noted, the levels for groups I and II being 850 and 806 I.U. per 100 cc., respectively.

When 9300 I.U. of vitamin A were administered daily to very small chickens, there was evidence of a toxic action. Although the average gains in weight of the surviving chickens in groups I and II were practically identical (136 and 127 gm., respectively), six of twenty-one chickens on a high vitamin A intake died before the termination of the experiment; these had grown only slightly and also showed an infection of the mucous membrane of the eye. In all cases the animals which showed these symptoms were the birds which were the lightest

in weight at the start of the tests. That the larger birds were not affected is shown by the fact that some of the largest gains in weight were scored by the group receiving the high vitamin A supplement.

SUMMARY

The lowering of the carotenoid pigments in the blood and livers of chickens following the administration of a high vitamin A diet is greater than that in the blood or milk of cows on a similar dietary regime. This is not caused by the failure to absorb similar amounts of the carotenoids, but must be attributed to the more rapid destruction.

The main pigments in the chickens are carotenols. Failure of the chicken to store carotene is not due to lack of absorption of this substance, but must be attributed to its transformation to other non-chromogenic compounds.

There is evidence of a toxic action of large doses of vitamin A, in the form of shark liver oil, to the 2-week-old chickens. This toxic action was only noted in the smallest birds.

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CHOLINE AND PYRIDOXINE AS FACTORS IN PREVENTION OF EPITHELIAL HYPERPLASIA IN THE FORESTOMACH OF RATS FED WHITE FLOUR

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(Received for publication July 31, 1942)

Young rats fed a diet of white flour, butterfat and salts develop hyperplasia of the forestomach epithelium. Since our last report, Fridericia and co-workers ('40) have described experiments in which they show that vitamin A deficiency may increase the incidence of stomach lesions although their etiology is related in a fundamental way to undetermined factors. Production of similar changes is influenced by the physical character of the diet but Chen ('41) was unable to determine the manner of action. Whether the lesions result directly from an action of the deficiency on the epithelial cells or indirectly from some other disturbed physiological process, they appear to be definitely related in the white flour diet to a multiple deficiency of the members of the vitamin B complex. Previous studies (Sharpless, '40) have shown that epithelial changes can be prevented in most rats fed white flour by supplements of riboflavin, nicotinic acid, cystine and an extract of rice polishings. These supplements alter neither the physical character of the diet, the vitamin A intake, nor the chronic starvation experienced by the rats. This is the report of studies in which thiamine, pyridoxine,[1] choline and calcium pantothenate [1] have been substituted for the rice polish extract.

[1] The pyridoxine and calcium pantothenate for this experiment were supplied through the courtesy of Merck and Co., Inc.

EXPERIMENTAL PART

Experimental procedures described previously (Sharpless, '40) were followed in this study. The basal diet had the following percentage composition: white flour 90.0, calcium lactate 2.9, sodium chloride 2.0, ferric citrate 0.1, butterfat 5.0. In addition, each kilogram of diet contained 10 mg. of thiamine chloride, 4 mg. of riboflavin, and 15 drops of viosterol. Experimental diets were composed of the basal diet with supplements added to each kilo as follows: diet 284 contained 20 mg. pyridoxine, diet 307 contained 1.5 gm. choline hydrochloride, diet 308 contained both pyridoxine and choline, and diet 310 contained 10 mg. of calcium pantothenate in addition to choline and pyridoxine.

RESULTS

The results are recorded in table 1. Sixty-three per cent of the rats fed the basal diet had one or more gastric lesions, whereas 85% of 151 rats (Sharpless, '40) had stomach changes when supplements of cystine, riboflavin, nicotinic acid and thiamine were omitted. Pyridoxine alone (diet 284) did not improve the diet; choline hydrochloride alone (diet 307) protected all but 31% of the animals. When fed together to fifty-eight rats, choline and pyridoxine (diet 308) reduced the incidence of lesions to 14%. Of the eight rats which had

TABLE 1

Number of animals, growth and incidence of gastric lesions.

DIET	NUMBER OF ANIMALS			AVERAGE WEIGHT CHANGE	
	Total	With lesions	Per cent with lesions	Rats with lesions	Rats without lesions
				gm.	*gm.*
Basal mixture	19	12	63	—2	—4
No. 284: basal + pyridoxine	49	31	63	0	0
No. 307: basal + choline	51	16	31	—2	0
No. 308: basal + pyridoxine and choline	58	8	14	+5	+9
No. 310: basal + calcium pantothenate + pyridoxine + choline	42	6	14	+7	+9
Stock diet (intake limited)	10	0	0	...	+1.6

macroscopically visible changes only one had more than two small papillomatous areas. Either sufficient pantothenic acid for the needs of these rats is contained in white flour or this dietary essential is not concerned in the development of gastric changes (diet 310).

Slightly better growth was obtained with those diets that provided the most complete protection. That this slight superiority of diet had little effect on the gastric phenomena here studied is evident when the results are examined critically. In any one diet the growth of rats that had abnormal and those that had normal stomachs differed but little. Previous studies (Sharpless, '40) demonstrated that certain diets stimulated growth without affecting the stomach pathology. In the present study ten rats, kept at constant weight for 6 weeks by limiting their intake of stock diet, had normal stomachs. The dietary restriction in these experiments, therefore, appears to be a factor in the development of changes only in so far as it limits intake of essential protective factors.

The results as a whole indicate that pyridoxine in the presence of choline stimulates growth and aids in maintaining normal gastric epithelium. In the individual groups that are included in the total for any one diet, this was not so clearly demonstrated. Of five groups fed diet 307, two could not be distinguished from the groups fed diet 308. However, uniform results were obtained in rats fed diet 308. The variation in results can be explained if it is assumed that pyridoxine concentration in different lots of white flour was not constant.

DISCUSSION

According to Fletcher, Best and Solandt ('35) white flour contains considerable choline. That the content is insufficient for rats when they have no other source of protein is demonstrated by these experiments. In our previous report it was suggested that the lesions result from a change in the response of the epithelial cells to proliferative stimulation along with the presence of a stimulating substance acting in the stomach. Many of the animals fed the basal diet or similar diets without

choline had intestinal contents in the stomach. Preliminary studies have shown that taurocholic acid increases the incidence and severity of the lesions and indicates that regurgitated bile may act on the gastric epithelium as a proliferative stimulus. All of the animals fed choline had a slight diarrhea and their stomachs were free from visible intestinal contents. Choline may stimulate the smooth muscle of the intestinal tract (Winfield, '38) and thereby help prevent stasis and regurgitation of intestinal contents into the stomach. Bulky and absorbent material should act as an effective mechanical barrier to regurgitation of bile and also stimulate intestinal movements. This would explain the observations made by Chen ('41) that the physical character of the diet is an important factor in the etiology of the lesions.

A second action of choline may be to supply available methyl groups. In two types of diets (Sharpless, '37, '40) cystine aids in maintaining normal gastric epithelium. Available methyl groups like choline and methionine (Griffith, '41) are necessary to prevent the toxic effect of cystine on kidneys of young rats; this suggests that choline in this diet might be required for the proper utilization of cystine.

Our previous observation that nicotinic acid, riboflavin and a sulfur-containing amino acid are related to the resistance of epithelial cells of the stomach to proliferative stimulation is similar to that reported by Kensler and co-authors ('41) that riboflavin plays some role in promoting the resistance of liver cells to the carcinogenic action of dimethylaminoazobenzene.

That pyridoxine is an essential factor in the prevention of stomach changes is not surprising. A number of reports have shown that riboflavin deficiency symptoms are not relieved by supplying this vitamin alone when pyridoxine is also lacking. Furthermore, Antopol and Unna ('39) observed extreme hyperkeratosis in the epithelium of the ears, paws and snout of rats in the extreme depletion stage of vitamin B_6.

SUMMARY

Choline and pyridoxine are necessary to help maintain normal epithelium in the forestomach of rats fed diets in which cystine and white flour furnish the only source of protein. Calcium pantothenate does not improve such diets. Using results of preliminary work with taurocholic acid as a basis, it is suggested that regurgitated bile in the stomach may be an important cause of the lesions. It is also suggested that choline helps prevent regurgitation of bile by stimulating the smooth muscle of the intestinal tract.

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GOITROGENIC ACTION OF CALCIUM AND VITAMIN D

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(Received for publication July 31, 1942)

In goiter surveys and experimental studies, calcium has been suggested as a goitrogenic agent. Surveys by Orr ('31) and Stott ('32) have shown that goiter often occurs in limestone regions. It is now commonly accepted that the thyroid influences calcium metabolism. However, there is no general agreement on the goitrogenic role of calcium in the reported experimental studies. Thompson ('36) found that the thyroid size varied directly with the calcium concentration of the diet, but Levine, Remington and von Kolnitz ('32) with the same low iodine diet observed no effect from calcium. Although Hibbard ('33) and Mahorner ('37) obtained a goitrogenic effect from calcium chloride, Hibbard concluded from studies with sodium chloride that chloride and not calcium was the cause of enlarged thyroids.

Experimental goiter in nearly all reports has been of the hyperplastic type. Hellwig ('35) introduced a new problem by asserting that calcium with a low iodine intake produced a hyperplastic thyroid and that a slight increase in iodine intake caused a colloid goiter. Both types of goiter were prevented by further increasing the iodine intake.

If calcium is goitrogenic it should be possible to demonstrate the fact experimentally with a diet that is known to produce a slight thyroid enlargement. It is assumed that

under these conditions the thyroid would be sensitive to substances with only slight goitrogenic action. This is the report of such a study in which the goitrogenic effect of three calcium salts (chloride, phosphate and lactate) was determined. Also reported is a study of Hellwig's diet of corn meal and rolled oats, undertaken to determine whether results similar to his could be obtained under our experimental conditions.

When the results of the above investigation were found to be negative, a study was made of the influence of vitamin D on the alleged goitrogenic action of calcium. According to Abelin ('28) calcium decreases the metabolic effect of thyroxine and therefore should increase the requirement. If calcium should act in this manner, vitamin D, through its action on absorption, would be as important as the calcium intake.

EXPERIMENTAL

The experimental conditions were the same as those previously described (Sharpless, Pearsons and Prato, '39). Both male and female rats, 4 to 5 weeks old, were used. The sexes were kept separate and two or three animals were placed in each cage. Littermates were distributed through the diets as much as possible. Of the three calcium salts studied, the chloride was prepared from calcium oxide and hydrochloric acid. The lactate and phosphate were fed without purification but a correction was made for the iodine they contained so that the total iodine added was 4.0 μg. per 100 gm. of diet. The basal diet had the following percentage composition: Soybean flour (unprocessed) 75.0, dried brewers' yeast 3.0, calcium carbonate 0.5, sodium chloride 1.0, butterfat 5.0, sucrose 15.5, and 4.0 μg. of iodide as potassium iodide. Two per cent calcium chloride or an equivalent amount of calcium in the other salts was added to the basal diet in place of an equal weight of soybean flour. In the experiment with calcium in the water, 1% calcium chloride was added to the drinking water. Hellwig's diet had the following percentage composition: Corn meal 50.0 and rolled oats 50.0. Two per cent calcium chloride was given to these animals in their drinking water.

RESULTS

The thyroid weight, iodine content and concentration for rats fed the different calcium salts are recorded in table 1. Littermate controls were fed the basal diet in all of the studies and the results were sufficiently uniform to combine them in one group. The thyroid weight per 100 gm. of body weight of these animals was 17 mg. which is approximately twice the normal thyroid weight and one-half the weight obtained when the diet is not supplemented with iodine. None of the

TABLE 1

Effect of calcium salts on weight and iodine content of the thyroid.

DIET	NO. OF RATS	THYROID WEIGHT		THYROID IODINE	
		Ave. total	Per 100 gm. body wt.	Total per gland	Dry basis
		mg.	*mg.*	*μg.*	%
Basal	212	22	17 ± 0.21	1.5	.026
1% $CaCl_2$ in drinking water	24	21	17 ± 0.7	1.7	.025
2% $CaCl_2$ in basal diet	71	23	17 ± 0.47	1.6	.029
6% calcium lactate	15	18	18 ± 0.8	1.2	.026
2% calcium carbonate	23	25	17 ± 0.97	1.2	.020
2% $Ca_3(PO_4)_2$	11	20	19 ± 0.8	1.8	.030
Rolled oats 50.0% Corn meal 50.0%	12	12	14 ± 0.5	3.3	.11
2% $CaCl_2$ in drinking water	16	16	12 ± 0.5	2.1	.06

calcium salts produced a real enlargement of the thyroid. The slight enlargement obtained with calcium lactate and calcium phosphate proved to be insignificant by mathematical analysis. The iodine concentration on the dry weight basis was almost identical for all four diets.

The diet composed of 50% corn meal and 50% rolled oats (Hellwig's diet, table 1) was unsatisfactory for growth. Although Hellwig does not state that rats fed this diet need any vitamin D supplement, the rats showed tetany and died within 4 or 5 weeks unless vitamin D was given. The usual dosage of 15 drops of viosterol per kilogram of food proved ineffective, but 2 drops per rat per day maintained life and

stimulated some growth in those getting no calcium chloride. The diet containing calcium chloride was supplemented with 3% of dried yeast after 6 weeks in order that the rats might survive. Yeast contains only a small amount of iodine and good results have been obtained with it in soybean goitrogenic diets. Only those animals which lived for the full 12 weeks are recorded in the table. Rats given calcium chloride had an average thyroid weight of 12 mg. per 100 gm. of body weight compared with 14 mg. for their littermate controls. The difference is not statistically significant and because the parathyroids of rats fed the low calcium diet were very large, the difference is not so great as the figures imply. Because of their intimate relation with the thyroid, the parathyroids must be weighed with it.

Examination of some glands from each group showed no real differences in the microscopic structure. Some of the glands from animals fed calcium chloride appeared more hyperplastic than those of littermate controls, but the differences were slight. The 2% calcium chloride fed in the diet is a large amount when compared with any normal diet. Because of this large amount of calcium, the slight hyperplasia obtained, while it supports the results of Mahorner with dogs, is believed to have no real significance from the standpoint of goiter incidence.

These results demonstrate that a high calcium intake does not affect the size of the thyroid. However, if calcium should influence the oxidation processes that are controlled by the thyroid the effect would be obtained from its action within the body and the factors that influence calcium metabolism should receive consideration. One of the principal factors affecting absorption and utilization of calcium is vitamin D.

The results of experiments with small and large amounts of vitamin D are shown in table 2. In the basal diet without iodine supplement, viosterol in either small or large amounts had no real effect on the size or iodine content of the thyroid. Calcium carbonate either alone or along with 2% viosterol caused no difference in iodine concentration of the glands and

TABLE 2

Effect of viosterol and calcium on weight and iodine content of the thyroid.

BASAL DIET SUPPLEMENT	NO. OF ANIMALS	BODY WEIGHT		THYROID WEIGHT		IODINE IN GLAND	
		Initial	Final	Ave. total	Per 100 gm. body wt.	Ave. total	Dry basis
		gm.	*gm.*	*mg.*	*mg.*	*μg.*	%
Basal —40 μg. I_2 per kg.	36	65	160	53	33 ± 1.1	0.7	.007
15 drops viosterol per kg.	29	68	131	44	34 ± 1.4	0.9	.009
2% viosterol	39	60	147	50	34 ± 1.1	0.6	.006
2% $CaCO_3$	17	73	145	55	37 ± 2.1	0.7	.007
2% $CaCO_3$ + 2% viosterol	17	70	134	49	37 ± 1.2	0.6	.006
Basal	82	65	139	23	17 ± .40	2.2	.038
I_2 + 2% viosterol	28	61	133	24	18 ± .72	2.3	.043
I_2 + 2% $CaCl_2$	70	65	128	23	18 ± .47	2.0	.038
I_2 + 2% $CaCl_2$ + 15 drops viosterol	55	67	141	28	20 ± .52	2.0	.033
I_2 + 2% $CaCl_2$ + 2% viosterol	27	63	134	29	22 ± 1.0	1.62	.029
I_2 + 2% $CaCO_3$	23	72	145	25	17 ± .97	2.2	.039
I_2 + 2% $CaCO_3$ + 2% viosterol	24	69	128	21	16 ± .63	2.0	.040

only a slight enlargement which was not mathematically significant. In the basal diet, however, both concentrations of viosterol produced an increase in thyroid weight when calcium chloride was fed, but no effect with calcium carbonate. The average thyroid weights per 100 gm. of body weight of rats fed the diets containing calcium chloride with either 15 drops or 2% viosterol were 20 mg. and 22 mg., respectively; mathematical analysis showed that, because of the greater individual variation in the latter group, both had the same significance, viz., a "P" value of .00 (Fisher, '34).

While 15 drops per kilogram of diet had no effect, 2% viosterol raised the average serum calcium from 12.6 to 13.3 mg. per 100 cc.

DISCUSSION

The two most evident facts in the results are (1) that elevated serum calcium has no tendency to increase the thyroid

weight, and (2) that calcium chloride, the salt that has been used in many experimental studies, has a significant goitrogenic effect when vitamin D is present.

Hibbard's ('33) results in which calcium chloride but not other calcium salts was found to produce goiter, are therefore confirmed. However, Hibbard's second conclusion that chloride is goitrogenic is not supported by these results since without vitamin D, calcium chloride did not cause an increase in thyroid weight.

When thyroid enlargement occurs and an increase in the iodine intake brings about a normal gland, the accepted explanation is that a relative iodine deficiency exists. The principal, if not the only, site of iodine storage is in the thyroid gland. Therefore, the iodine content of the thyroid gives a direct measure of the amount of iodine stored in the body and is a more reliable indication of the iodine balance than its determination from the food and excreta. The thyroids of rats at the start of the experiment contained approximately 2.0 μg. of iodine or 0.13% of dry weight. From the results it can be seen that with the exception of diets lacking iodine supplement the net iodine balance for the experiment is practically zero. Furthermore, it can be calculated that a 150-gm. rat with a normal thyroid weight of 0.0105 gm. and an iodine concentration of 0.1% of the dry weight would contain 2.1 μg. of iodine. Since glands with an iodine concentration of 0.1% dry weight have a normal microscopic appearance, it must be concluded that within the limits of 1.6 to 2.3 μg. (table 2) the total is within the normal range. Since additional iodine in the diet will produce an approximately normal gland, these results are interpreted as evidence of a relative iodine deficiency. Balance studies which have been proposed for this experiment could indicate only the summation which is really a determination of absolute iodine deficiency.

A relative iodine deficiency could occur (1) when there is an excessive excretion of iodine before it can be utilized, (2) when the requirement for thyroxine is increased, or (3) when the mechanism for thyroxine formation is blocked. All of these conditions assume that the iodine is absorbed in

available form. While the iodine content of the glands mentioned above is within the calculated normal range for these animals, the iodine concentration is low. Chlorides tend to decrease the concentration and the greatest reduction occurred when calcium chloride was fed with 2% viosterol. Growth and food intake were similar in all diets. Iodine storage was slightly less in rats fed calcium chloride and 2% viosterol, which suggests that it could have been excreted before being utilized. Other data not given here demonstrate that chloride can cause a reduction in the content and concentration of iodine in the thyroid.

The possibility that viosterol, by way of its alleged effect on metabolism (Deutsch, Reed and Struck, '36), might influence the results is ruled out by control experiments in which it did not influence the size, iodine content or iodine concentration of the thyroid.

Hellwig's ('35) hypothesis that goiter is caused by a positive factor along with an iodine deficiency is partially supported by these results, namely, that an apparent decrease in available iodine occurred when calcium chloride produced thyroid enlargement. Because calcium chloride was the only salt that was goitrogenic, neither of its components should be classed as having this property. Furthermore, the compound should be considered as only a potential goitrogenic agent.

The fact that calcium carbonate plus 2% viosterol caused a slight but not significant decrease and calcium chloride caused an increase in thyroid weight raises the question whether acidosis might play a role in thyroid enlargement. Experiments on this point have not provided consistent results. Direct determinations were not made but some evidence is provided in these results. Calcium chloride without vitamin D had no significant goitrogenic effect. Vitamin D causes a shift in calcium excretion from the feces to the urine, thereby increasing the acidity of the feces. It is assumed that this would tend to relieve a condition of acidosis if it was present, yet calcium chloride was goitrogenic only when vitamin D was given. Loose stools were common in rats receiving calcium chloride but extensive diarrhea was not observed.

Other experiments not reported here show that inclusion in the basal diet of hydrochloric acid, equivalent to 70% of the acid producing effect of 2% calcium chloride, had no significant effect on the thyroid weight.

SUMMARY

The purpose of these experiments was to determine whether calcium could be classed as a goitrogenic substance. When other factors are kept constant, all of the evidence indicates that it does not influence the size of the thyroid gland. However, along with vitamin D calcium chloride can act as a goitrogenic agent. While the mechanism of this action cannot be absolutely determined from these experiments, the results suggest that chloride causes some loss of iodine which is followed by an increase in thyroid weight when an excess of calcium is absorbed.

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THE CRITICAL TEMPERATURE FOR THE ALBINO RAT AS AFFECTED BY FEEDING [1]

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ONE FIGURE

(Received for publication September 8, 1942)

The prevailing understanding that the critical temperature of the environment for an animal receiving food is lower than for the same animal without food, as expressed in text and reference books, rests upon an experiment on dogs (Rubner, '02), which the authors of the present paper consider inconclusive, and upon records of food consumption and animal production in experiments (Sanborn, 1892; Waters, '07; Mairs and Tomhave, '08; Armsby, '08; Buckley, '13; Davis, '13–'14; Woodward et al., '18; Potter and Withycombe, '21; Dice, '40) which show that fattening steers and well-fed milch cows produce as economically in an open shed, with protection from wind and rain, as in a warm barn.

While the authors of this paper acknowledge the logic of the principle that heat increments due to the food lower the critical temperature of the environment for animals, as determined during fast, this generalization depends upon the constancy of certain background conditions which may conceivably vary. Information, therefore, is needed as to the details of application of this principle to the albino rat since this species is so much used in metabolism investigations, so frequently under conditions other than fast.

[1] Authorized for publication on September 2, 1942, as paper no. 1123 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

The objectives of the work here reported, therefore, were (a) to establish the critical temperature for the albino rat receiving feed, and (b) to determine the percentage of the total heat production which is eliminated as latent heat of water vapor.

EXPERIMENTAL

Fourteen mature male albino rats of approximately the same age were established on a commercial dog feed [2] in amounts slightly more than the maintenance requirement, but close to the maximum that the animals would consume. The daily allotment of 15 gm. of feed was divided into two equal portions and fed at 7:30 A.M. and 5 P.M. After several days on this schedule, the animals cleaned up their feed quickly and the experiment was then started. The feed intake remained constant throughout the experiment, which was in progress for approximately 3½ months.

The apparatus used was constructed in accord with the open circuit Haldane principle, the CO_2 and H_2O being determined gravimetrically, by absorption, and the oxygen by difference. Respiration measurements began between 8:00 and 8:30 A.M., and continued for 7½ hours. The respiratory quotient and the oxygen consumption, as determined, represent the entire 7½ hours, while the values for heat production and water vapor represent the last 6 hours of this period, the CO_2 and the H_2O vapor for the first 1½ hours being excluded in order to allow time for the animal to become quiet, and to permit the metabolism to come into adjustment with the prevailing temperature.

The physical activity of the subjects was controlled by conditions of management found satisfactory during long experience. The experiments were always conducted during the same hours of the day. The rats were kept quiet by a bright light and were watched during experiments. The CO_2 production was measured usually in 1- or 2-hour periods, thus permitting the exclusion of any period in which the animals would be active.

[2] Purina dog chow.

A complete description of the equipment used and of the procedure followed was given in a previous publication by Swift and Forbes ('39) reporting the determination of the critical temperature for the fasting rat. The same general procedure was followed in both experiments except that in the investigation now discussed the rats were established on a liberal plane of nutrition, while in the earlier study all determinations were made on fasting animals.

To prevent contamination of the water vapor with moisture evaporated from the urine and feces, a layer of paraffin oil about ½ inch deep was kept in the bottom of the respiration chamber. A floor above this oil, made of hardware cloth — two meshes to the inch — served as a support for the rat. The urine and feces as voided fell into the oil, and were immediately covered by it and thus protected from evaporation. A little urine unavoidably stuck to the screen floor and, if evaporated, would cause a slight error in the water determination, but no error in the total heat production. This error could not be of any considerable magnitude.

In order to permit direct comparison of the results with those of the earlier work by Swift and Forbes ('39) on fasting rats the values for heat production were computed as for a live weight of 325 gm., in accord with the two-thirds power of the body weights. They were also computed on the basis of 350 gm., as this was the approximate average live weight of the rats during the experiment; however, both bases supported exactly the same conclusions.

RESULTS

During the time this experiment was in progress, approximately 3½ months, all the rats gained slowly in weight, the average increasing from 327 to 383 gm. The results, therefore, represent rats in a normal state of nutrition, considering their age and weight.

The fact that the environmental temperature at which an animal has been living affects the rate of heat production has been demonstrated many times. In a recent study of meta-

bolic adaptation to temperature in the rabbit, Lee ('42) determined the minimum heat production, as measured at 28°C., in rabbits which had lived at 31°C. for 3 weeks.

In the present study the temperature of the colony room in which the subjects had lived was not thermostatically controlled, but was maintained, at most times, close to 25°C. The difference in temperature between this room and the metabolism chamber at thermal neutrality, therefore, was not great, but it is conceivable that the absolute minimum of metabolism was not reached. There is little reason, however, to believe that this would change the critical temperature; in fact, Lee did not find the critical temperature of the rabbit to be altered in a similar situation.

To prevent a possible cumulative effect of temperature changes on metabolism, the order of experimentation was arranged in such manner as to avoid a sequence of measurements during either ascending or descending temperatures; and this arrangement was also such as tended to avoid cumulative effects of advancing age of the subjects.

The final results are presented in table 1. The number of different animals used at each temperature is the same as the number of 7½ hour observations included in the table. The curve representing the course of the heat production with change of environmental temperature is given in figure 1 along with the curve for fasting rats as found by Swift and Forbes ('39).

The data show that the total heat production of the rats receiving feed decreased with increase in environmental temperature from 12° to 31°C., remained nearly constant through the temperature range from 31° to 33°C., and then rose sharply at 34°C. The odds expressing the significance of these values indicate clearly that the critical temperature as determined for the fasting rat was not lowered by the giving of feed.

A measure of the consistency of the heat production values at the several temperatures is afforded by an average coefficient of variation of 4.8% and an average probable error of

TABLE 1

Total heat production and water vaporized with change in environmental temperature.

ORDER OF EXPERIMENTS	AVE. LIVE WEIGHT	NUMBER OF 7½-HOUR OBSERVATIONS	TEMPERATURE	RESPIRATORY QUOTIENT		TOTAL HEAT PER HOUR			HEAT ELIMINATED BY VAPORIZATION OF WATER	
				Ave.	Coefficient of variation	Ave.[1]	Coefficient of variation	Odds of significance [2]	Per cent of total	Coefficient of variation
	gm.		*°C.*	%	%	*Cal.*	%			%
8	381	13	12	0.837	0.8	3.00	4.0		10.5	11.0
9	383	12	18	0.859	1.6	2.40	7.2	Billions to 1	12.8	15.7
7	373	13	24	0.902	1.3	1.79	4.3	Billions to 1	15.4	7.9
2	335	14	28	0.948	2.0	1.59	4.3	Billions to 1	18.8	11.6
1	327	14	30	0.946	1.1	1.50	5.3	1,052 to 1	20.2	8.8
5	366	14	31	0.951	1.2	1.38	2.3	14,700,000 to 1	21.3	9.3
3	354	14	32	0.942	2.3	1.38	2.0	0	21.5	8.5
6	373	13	33	0.915	2.7	1.45	7.9	31 to 1	29.1	18.6
4	362	14	34	0.918	2.2	1.61	6.1	13,912 to 1	48.8	13.2

[1] Computed to live weight of 325 gm.

[2] Odds that the heat production is significantly different from the one at the next lower temperature.

$\pm$ 0.016 for the series. These statistical values are almost identical with those found for the fasting rat by Swift and Forbes ('39).

While the curves of heat production of the fed rats and the fasting rats (fig. 1) were not determined on the same animals, or at the same time, they were determined on rats of the

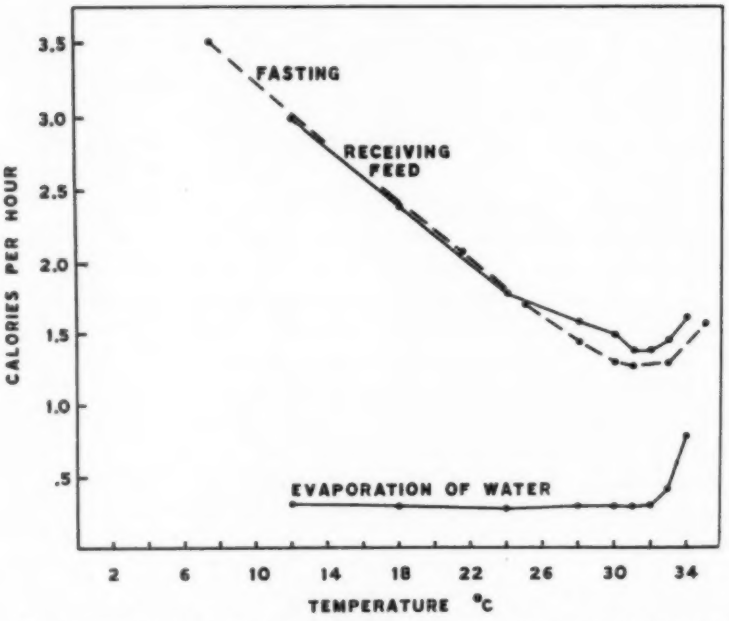

Fig. 1 Comparison of the heat production of fasting albino rats with that of animals receiving feed at different environmental temperatures, and the heat disposal by evaporation of water in the feeding experiment.

same closely inbred stock which had been produced by the same method of feeding under essentially the same general environmental conditions.

The proportion of the heat eliminated as latent heat of water vapor (table 1) increased steadily and rapidly with rise in environmental temperature, but the amount of heat so eliminated (fig. 1) remained virtually constant during rise in

environmental temperature from 12°C. to 32°C. With further rise in environmental temperature there was a sharp increase in the amount of heat eliminated as latent heat of water vapor.

As far as the authors are aware, this is the first recorded experiment in which feeding did not appear to lower the critical temperature as determined during fast. This finding was not anticipated, as a contrary understanding has been expressed in the literature for many years.

In an effort to explain the results observed, the writers cite the following facts: (1) the heat increment of such a mixed diet as was employed was relatively low, as compared with heat increments due to single nutrients, especially protein, as demonstrated by Forbes, Bratzler, Thacker and Marcy ('39); (2) the feed consumption of the rat is relatively small, as compared with that of domestic animals which have been selectively bred for high production, necessitating high feed consumption; (3) the heat increment of the food of the monogastric rat is small as compared with that of polygastric ruminants, in the alimentary tracts of which fermentation with its attendant heat production is so prominent a condition; (4) it is not known that the rat is perfectly homeothermic; and (5) the rat possesses a degree of adaptation to heat loss through hunching up when cold, and stretching out when overheated, which must serve to lower the critical temperature and to raise the point of hyperthermal rise — thus widening the zone of thermal neutrality.

The greatest effect, if any, of feed on the critical temperature should be manifest under conditions of most abundant food intake. To obtain additional evidence on this point, young rapidly growing rats were used during the period of their maximum feed consumption. Two 8-hour measurements of the heat production were made at each of three temperatures. The rats, averaging 125 gm. in weight at the time the measurements were made, were given 10 gm. of feed daily in most cases, and were gaining at the rate of 2.6 gm. per day. The heat production of the rats was measured at one of the three

temperatures on each of 3 consecutive days, the temperature order being reversed, when this was possible, with each rat.

The heat production, computed to a live weight of 125 gm., in proportion to the two-thirds power of the weight, is given in table 2. The evidence is again conclusive that the critical temperature under these conditions was at least as high as 30.5°C., the critical temperature for the fasting rat.

TABLE 2

Heat production of young rats on full feed as affected by change of environmental temperature.

NUMBER OF 8-HOUR OBSERVATIONS	TEMPERATURE	RESPIRATORY QUOTIENT	TOTAL HEAT PER HOUR	ODDS OF SIGNIFICANCE [1]
	°C.		Cal.	
10	20	0.871	1.44	
10	28	0.935	1.04	Billions to 1
10	30.5	0.948	0.91	13,912 to 1

[1] These odds represent the significance of the difference between each heat production value and the one at the next lower temperature.

As a factor of possible significance in connection with this investigation a test was made of the speed of ventilation of the respiration chamber. When the speed of ventilation of the chamber was varied between a rate of about 0.4 liter per minute, which gave a concentration of approximately 1% CO_2 in the respiration chamber, and a rate 5.25 times as fast, no consistent difference was found in the total heat production of fasting rats at the critical temperature. There was naturally some change, however, in the manner of heat disposal — more water being vaporized at the higher speeds of ventilation.

A part of the respiration work in connection with this problem was done with an apparatus which controlled the temperature of the respiration chamber by means of an air bath instead of a water bath as in the apparatus described by Swift and Forbes ('39). Of interest in this connection is the observation that when the air bath was maintained at a temperature of 28.2°C. the temperature inside the respiration chamber (a half gallon Mason jar) was 31.5°C. This fact

emphasizes the necessity of using the temperature inside the respiration chamber as that to which the animal is exposed. This observation may account for the higher critical temperature for the fasting rat as determined by Swift and Forbes ('39) in contrast to that reported by a number of other workers.

SUMMARY

The heat production of fourteen mature male albino rats receiving a constant feed intake was determined in 121 7½-hour periods of observation at environmental temperatures ranging from 12° to 34°C. Feeding did not lower the temperature of minimum heat production below 30°C. — the critical temperature for the fasting rat.

In another group of young rapidly growing rats, on full feed, the zone of thermal neutrality was still unchanged.

The prevailing opinion that feeding lowers the critical temperature, therefore, was not found true in relation to the albino rat under the conditions of experimentation.

At environmental temperatures below 25°C. the curves of heat production for rats receiving feed were approximately the same as for fasting rats, when computed to a basis of the same live weight.

At environmental temperatures above 25°C. the heat production of the rats receiving feed was higher than that of the fasting animals, as determined by the plane of nutrition and the dynamic effect of the feed.

The percentage of total heat eliminated as water vapor remained constant at temperatures from 12°C. to 32°C. With further rise in environmental temperature there was a sharp increase in both the amount and the percentage of heat eliminated as latent heat of water vapor.

It is not concluded from this investigation that the critical temperature of the environment for animals in general is unaffected by feeding.

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THE ROLE PLAYED BY THE GASTROINTESTINAL TRACT IN THE ABSORPTION AND EXCRETION OF RIBOFLAVIN

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(Received for publication September 18, 1942)

A good deal of attention has been given to the excretion of riboflavin by the kidney (Strong, Feeney, Moore and Parsons, '41; Axelrod, Spies and Elvehjem, '41; György, '42) but little is known about its excretion into the intestine and its absorption from the different parts of the gastrointestinal tract. This is perhaps not surprising since under ordinary circumstances it is difficult to tell what part of the riboflavin present in the alimentary canal has been synthesized by the intestinal flora and what part has entered it by either ingestion or excretion through the intestinal epithelium.

Riboflavin has a very characteristic yellow color and forms typical crystals when present in high concentration in an aqueous medium. Hence, an attempt was made to obtain additional information concerning its distribution in the body, by determining the presence of riboflavin crystals in various parts of the gastrointestinal tract following administration of the compound by the intravenous route or after direct introduction into isolated intestinal loops. Preliminary experiments have shown that if a saturated solution of riboflavin in 0.9% NaCl is injected intravenously in the rat the greater part of it reappears in the urine. This is easily demonstrated by the presence of innumerable crystalline concretions whose yellow color is readily detectable in the ureters and bladder within a few hours after the compound has been

injected into the blood stream. In order to make other pathways of excretion more readily detectable, all the experiments to be reported in this communication had to be carried out in bilaterally nephrectomized rats. It must be emphasized, therefore, that the conclusions derived from them are not necessarily applicable to the intact animal.

In nephrectomized rats riboflavin can be excreted into the upper part of the intestine

In the first experimental series twelve adult male albino rats weighing 130–170 gm. (average 155 gm.) were used. All animals were bilaterally nephrectomized by the dorsal approach under ether anesthesia. Immediately after this operation, while the animals were still under the influence of the anesthetic, each rat received an intravenous infusion of 7 mg. of riboflavin dissolved in 7 cc. of warm physiological saline. In half of the animals, the intestinal lumen had been occluded by placing a ligature at the transition between jejunum and ileum, thus dividing the intestinal tract into two separate compartments. Immediately after the injection the skin of all the animals exhibited the characteristic yellow color of riboflavin. Five hours later it was found, however, that while the skin of the animals with intestinal ligature was still yellow, that of the others became practically normal in color. At this time all animals were killed. Inspection of their intestines revealed no detectable evidence of riboflavin in the animals without intestinal ligature, while in the other group large amounts of the substance were detectable in the duodenum and jejunum but not in any other part of the alimentary canal. The characteristic yellow riboflavin crystals of the upper intestinal segments were readily identified under the microscope. This experiment was interpreted as indicating that riboflavin can be excreted into the upper part of the small intestine, and that if its downward movement towards the ileum and large intestine is prevented by a ligature, further excretion against a high concentration gradient in the upper

small intestine is prevented, or else the compound is continuously reabsorbed so that the organism cannot rid itself of it as readily as if passage through the intestine were free.

Excretion through the biliary system plays no important role in intestinal elimination of riboflavin: Most of the excretion is through the mucosa of duodenum and jejunum

A second experimental series was then performed to determine whether the riboflavin found in the duodenum is excreted through the bile or whether it is eliminated by the intestinal epithelium. The experiment was so conducted as to narrow down the site of elimination to a certain part of the intestinal canal, in the event that the compound is not excreted by the liver. Twelve adult male albino rats weighing 135–167 gm. (average 149 gm.) were nephrectomized. In six of these immediately after the nephrectomy the following interventions were performed. The bile duct was cut between two ligatures, another ligature being placed at the pylorus obstructing the communication between the stomach and the duodenum. Further ligatures occluded the transition between lower duodenum and jejunum and ileum, ileum and cecum, cecum and large intestine, the last ligature being placed on the rectum just above the anus. All these ligatures were so placed that they did not interfere with the blood supply to the various parts of the intestinal tract. Thus the gastrointestinal canal was subdivided into separate compartments consisting of the stomach, duodenum, jejunum, ileum, cecum and large intestine, respectively, the influx of bile into the intestine having been completely eliminated. Immediately following the operation the animals of both groups received an intravenous injection of 7 mg. of riboflavin dissolved in 7 cc. of warm physiological saline solution. All animals were sacrificed 5 hours after the injection. At autopsy riboflavin crystals were found in large quantities in the duodenum and jejunum in both groups of animals, while no trace of the compound was de-

tectable in the other parts of the intestinal canal. It was concluded that excretion through the biliary system plays no important role in the intestinal elimination of riboflavin and that most of the compound is excreted though the lining of the duodenum and jejunum. If some excretion through other parts of the intestinal tract did occur, it was overcompensated by rapid reabsorption or destruction.

Riboflavin is readily absorped from the lower part of the small intestine and excreted into the upper part

A third experiment was performed in order to study the disappearance of riboflavin introduced directly into various parts of the intestinal tract. Twelve male albino rats weighing 125–155 gm. (average 136 gm.) were nephrectomized, and in all of them a ligature was placed at the transition of the duodenum and jejunum. In six of the animals 7 mg. of riboflavin suspended in 0.5 cc. of saline was injected craniad from the ligature, while in the remaining six the same amount was introduced caudad from the ligature. In both cases the hole made by the injection needle was occluded by a small ligature placed tangentially on the intestinal wall. Sixteen hours later all animals were killed. In the group in which the compound had been injected craniad from the ligature riboflavin could be detected only in the cranial segment of the intestine, while in the other group it was present both above and below the ligature. The experiment shows that riboflavin is readily absorbed from the lower part of the small intestine and is then excreted into the upper part, while whatever excretion may occur into the lower part following absorption from the upper segment is not sufficiently important to be detectable in this type of experiment. This may be so either because too little is absorbed from the duodenum, or else, because whatever is absorbed and then excreted into the lower small intestine is so rapidly destroyed or reabsorbed here that it is not detectable by our technic.

Riboflavin is not efficiently absorbed but is rapidly destroyed in the large intestine

The fourth experimental series was designed to determine whether riboflavin is rapidly destroyed in the large intestine or especially quickly absorbed from it since no traces of the compound have ever been visible in this part of the intestine in the previous experiments. Six male albino rats were nephrectomized and immediately after the operation 7 mg. of riboflavin in 0.5 cc. of saline were injected into the cecum after the placing of one ligature at the ileo-cecal junction and another on the rectum, thus isolating the large intestine from the rest of the gastrointestinal tract. Sixteen hours later the animals were killed and no riboflavin was detectable in any part of the gastrointestinal tract. Since the same amount of this vitamin injected into the isolated ileum was followed by a definite excretion into the duodenum it appears that the compound is not efficiently absorbed but rapidly destroyed in the large intestine.

Excretion and destruction of riboflavin occurs largely through renal and intestinal tissue

In the last experiment the entire gastrointestinal tract was removed simultaneously with the kidneys in six male rats weighing 155–174 gm. (average 168 gm.). Intravenous administration of 7 mg. of riboflavin in 7 cc. of warm physiological saline showed that the vitamin is not noticeably destroyed in the absence of both liver and intestines as judged by the fact that the skin color did not fade during the course of this experiment. (The animals were killed after 24 hours.) Of course, this does not mean that in the absence of kidneys and intestine riboflavin cannot be destroyed, since our crude technic could not detect small changes in the riboflavin concentration of the tissues. It does indicate, however, that the major part of riboflavin excretion and destruction occurs through the renal and intestinal tissue.

SUMMARY AND CONCLUSIONS

Experiments in the rat indicate that following bilateral nephrectomy, intravenously administered riboflavin is rapidly excreted into the small intestine, especially the duodenum. Excretion through the bile plays no important part in the elimination of this vitamin. Destruction of riboflavin proceeds slowly, if at all, in an isolated loop of the duodenum, but quickly in the isolated large intestine. If riboflavin is directly injected into the isolated lower small intestine (jejunum and ileum) it is more rapidly absorbed from this site than it can be destroyed. Whether any destruction occurs in this segment is uncertain but the absorbed vitamin rapidly reappears in the duodenum.

In the complete absence of the intestinal canal and both kidneys the tissues of the rat cannot destroy or eliminate any significant percentage of large doses of intravenously injected riboflavin.

ACKNOWLEDGMENTS

The costs of this investigation were defrayed by the Hoffmann-LaRoche Company through the "Roche Foundation" allocated by its local representative, Mr. Paul Blanc. The author is also indebted to Dr. A. D. Odell who supplied the riboflavin used in these experiments.

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THE RETENTION OF VITAMINS IN MEAT DURING COOKING[1]

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(Received for publication October 19, 1942)

The vitamin content of meats has been reviewed by Waisman and Elvehjem ('41). Since most meats are cooked before consumption, there is considerable interest in the possible loss of vitamins during the preparation of these foods. Accurate information on this subject is very meager. In previous work conducted in our own laboratory, the methods of cooking and preparation of the samples were not properly standardized. We have, therefore, decided to make an extensive study of this problem and in this paper we wish to report the retention of thiamine, riboflavin, and nicotinic acid in samples of pork cooked under standard conditions.

EXPERIMENTAL

Samples of pork ham and loin were taken from carcasses weighing approximately 190 pounds. The loins and hams were obtained from the same hogs at a local packing plant. Paired cuts from the right and left loins and hams were prepared leaving about $\frac{1}{2}$ inch of fat on the outer surface. Loin cuts containing the last three thoracic and the first three lumbar vertebrae were taken for the roast, and 1-inch cuts were

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Supported in part by a grant from the National Livestock and Meat Board, made through the National Research Council.

removed from each end of the remaining loin for braising and broiling. Ham cuts 1-inch thick were removed parallel to the aitch-bone for broiling. Then 3-inch ham cuts for roasting were removed adjacent to the broiling cuts and on the shank end. Corresponding cuts from the opposite ham and loin were used for fresh and cooked samples.

The cooking was carried out under standard procedures, without seasoning, using the methods of cooking described in the publication ('33) of the United States Department of Agriculture and State Agricultural Experiment Stations. Fresh loin cuts were roasted, braised and broiled and fresh ham cuts were roasted and broiled. Loin samples were braised as follows: The loin cuts 1-inch thick were placed in a pan which had been pre-heated for 2 minutes on an electric stove. The chops were browned on each side for 2½ minutes at moderate heat and then placed on a rack and covered. The rest of the braising procedure was carried out at low heat until the internal temperature of the meat reached 87°C. The cooking time varied with the size of the cut. In our specific studies the range in cooking time for roast loins was from 2 hours to 2½ hours and for roast ham from 3 to 3½ hours. Approximately 45 minutes were required for braising loin cuts, 30 minutes for broiling loin cuts, and 45 minutes for broiling ham cuts.

The bones were removed and weighed after cooking. The net weights of the fresh and cooked meats were determined by subtracting the weight of the bone, which was found not to change in weight significantly during cooking.

Both the fresh and cooked samples were ground three times through an electric meat grinder and thoroughly mixed to assure homogeneity. All meats were stored in dark glass containers in a —4°C. cold room. Duplicate moisture and fat determinations were made on all samples. The moisture was determined by drying 10-gm. samples of meat at 50°C. for 48 hours. The fat content was determined on the residue by extracting with ether in a Caldwell extractor for 16 hours.

The per cent solids were calculated by subtracting the per cent of fat and water from 100.

The drippings were collected from all cooked samples. They were then extracted with 0.1 N sulfuric acid by heating on a steam bath with continuous stirring for 15 minutes. The aqueous layer was retained for vitamin analysis.

The thiamine content of the samples and drippings was determined by the method of Hennessy ('42), with slight modifications in the extraction procedures. Two and one-half gram samples of meat were treated with 75 cc. of 0.1 N sulfuric acid at 70°C. for 1 hour and then digested with 0.3 gm. of clarase in 5 cc. of 2.5 molar sodium acetate for 1 hour at 50°C., and an additional 12 hours at 37°C. in an incubator. The samples were then filtered and made up to volume in 100 cc. volumetric flasks and carried through the rest of the procedure as directed in the method.

The microbiological method of Snell and Strong ('39) was used for the riboflavin assays with modifications in the extraction procedure. Two-gram samples of meat were homogenized with 0.1 N sulfuric acid, then 0.2 gm. of clarase and 0.2 gm. of papain were added in 5 cc. of 2.5 molar sodium acetate and placed in an incubator at 37°C. for 24 hours. The samples were then autoclaved, neutralized and filtered. The filtrates were extracted with ether (Strong and Carpenter, '42) and the aqueous layers were transferred to 100 cc. volumetric flasks and made up to volume. Suitable aliquots were transferred to tubes for analysis. The amount of riboflavin found in papain was negligible, but clarase was found to contain 0.45 μg. of riboflavin per gram. Since 0.1 gram of clarase was added per gram of meat, corrections of the riboflavin values were made.

The nicotinic acid content was determined by the microbiological method of Snell and Wright ('41) using a 4% NaOH digestion.

Analyses have been completed on a total of seven different series of samples and the detailed results of one of the series are given in table 1.

TABLE 1

Detailed analyses of meats. Seven different series of samples were analyzed. Illustrative results of the second series.

	FRESH PORK LOIN ROASTED	FRESH PORK LOIN BRAISED	FRESH PORK LOIN BROILED	FRESH HAM ROASTED	FRESH HAM BROILED
Before cooking					
Fresh weight (gm.)	1170	332	321	2101	942
Water content (gm.)	560	162	159	1215	514
Fat content (gm.)	401	115	106	486	260
Total solids (gm.)	209	54	58	400	169
Thiamine content (mg.)	15.7	4.2	4.5	29.6	13.9
Riboflavin content (mg.)	2.96	0.90	0.93	6.82	3.06
Nicotinic acid (mg.)	44.5	12.1	12.0	65.2	25.8
After cooking					
Weight after cooking (gm.)	913	232	232	1669	664
Water content (gm.)	394	81	100	830	309
Fat content (gm.)	330	99	75	470	200
Total solids (gm.)	189	52	57	370	155
Thiamine content (mg.)	11.0	2.3	3.5	23.7	10.0
Thiamine in drippings (mg.)	0.1	0.54	0.26	1.20	1.23
Riboflavin content (mg.)	2.69	.67	.78	5.76	2.56
Riboflavin in drippings (mg.)	.09	0.13	.08	0.36	0.24
Nicotinic acid content (mg.)	37.4	7.3	11.2	58.6	20.9
Nicotinic acid in drippings (mg.)	1.65	2.75	1.32	4.74	3.46

All of the vitamin analyses were made on fresh and cooked meats. The per cent retained in the meat alone was calculated by dividing the total milligrams of the vitamin in the entire piece of cooked meat by the total milligrams in the uncooked meat. The total per cent retained was calculated by adding the milligrams of vitamin in the drippings and fresh cooked meat and dividing the sum by the total milligrams in the uncooked meat.

The per cent of the vitamins retained in the meats alone and the total per cent retained in the meat plus drippings from four series of analyses are shown in table 2.

TABLE 2

Vitamin retention after cooking. All values in per cent.

	DETERMINATION	ROAST LOIN	BRAISED LOIN	BROILED LOIN	ROAST HAM	BROILED HAM
Thiamine	1	64	49		62	
retained	2	70	54	79	80	72
in meat	3	65	49	66	66	72
	4	73	49	55	66	72
Total	1	67	61		66	
thiamine	2	71	67	85	84	82
retained	3	70	71	72	68	75
	4	76	73	57	68	79
Riboflavin	1	76	72		79	
retained	2	90	75	82	84	84
in meat	3	100	81	90	86	81
	4	95	80	75	79	82
Total	1	85	85		86	
riboflavin	2	93	90	91	87	91
retained	3	106	102	100	92	88
	4	101	102	88	85	90
Nicotinic	1	74	63		89	
acid	2	84	63	93	90	81
retained	3	77	61	73	98	82
in meat	4	82	74	73	81	82
Total	1	82	100		103	
nicotinic	2	88	84	104	97	95
acid	3	84	85	75	102	96
retained	4	88	102	87	94	95

The vitamin content per gram of dry meat was calculated from the determined values of the fresh meat. The values are summarized in table 3.

DISCUSSION

It should be pointed out that in previous work (Waisman and Elvehjem, '41), most of the surface fat was removed before analysis. Our figures for the vitamin content in micrograms per gram of fresh meat, therefore, cannot be compared directly to earlier work. Thiamine and nicotinic acid values

TABLE 3

Table of analysis of fresh and dry samples.

	SAMPLE NO.	THIAMINE		RIBOFLAVIN		NICOTINIC ACID	
		μg./gm. fresh	μg./gm. dry	μg./gm. fresh	μg./gm. dry	μg./gm. fresh	μg./gm. dry
Fresh loin	202	7.4	13.4	1.6	2.9	31	56
Roast loin	203	6.7	11.9	1.7	3.3	32	57
Fresh loin	210	13.4	23.0	2.6	5.0	38	79
Roast loin	211	12.0	21.1	3.0	5.3	41	72
Fresh loin	220	9.6	21.8	2.3	5.3	48	109
Roast loin	221	7.9	14.9	2.9	5.5	47	89
Fresh loin	230	15.2	33.9	2.8	6.2	35	78
Roast loin	231	14.2	29.4	3.4	7.0	37	76
Fresh loin	202	7.4	13.4	1.6	2.9	31	56
Braised loin	204	5.1	7.4	1.6	2.3	30	44
Fresh loin	212	12.8	25.0	2.7	5.3	37	72
Braised loin	213	9.9	15.3	2.9	4.5	32	78
Fresh loin	222	9.9	22.4	2.3	5.2	47	106
Braised loin	223	7.0	11.8	2.6	4.3	40	67
Fresh loin	232	15.0	33.2	3.0	6.7	39	84
Braised loin	233	10.0	17.1	3.3	5.6	39	67
Fresh loin	214	14.0	27.6	2.9	5.7	38	75
Broiled loin	215	15.6	27.4	3.4	6.0	49	86
Fresh loin	224	10.1	22.8	2.4	5.4	49	108
Broiled loin	225	10.0	18.4	3.2	5.9	54	99
Fresh loin	234	13.2	27.6	3.0	6.3	36	75
Broiled loin	235	14.2	26.0	4.4	8.0	52	95
Fresh ham	206	7.7	17.8	2.1	4.9	35	81
Roast ham	207	6.7	14.1	2.3	4.9	43	91
Fresh ham	216	14.1	33.5	3.3	7.8	31	74
Roast ham	217	14.1	28.0	3.5	7.0	35	70
Fresh ham	226	9.1	24.0	3.1	8.2	35	93
Roast ham	227	7.6	17.1	3.8	8.4	38	84
Fresh ham	236	13.5	36.2	3.6	9.6	38	101
Roast ham	237	13.5	26.7	4.3	8.5	47	93
Fresh ham	206	7.7	17.8	2.1	4.9	35	81
Braised ham	208	5.1	8.5	2.2	3.7	36	60
Fresh ham	218	14.8	32.6	3.3	7.3	31	68
Broiled ham	219	14.8	27.6	3.9	7.3	32	60
Fresh ham	228	9.2	21.2	2.9	6.7	32	74
Broiled ham	229	10.4	19.7	4.0	7.6	41	78
Fresh ham	238	13.0	29.0	3.4	7.7	36	80
Broiled ham	239	14.8	27.3	4.4	8.1	47	87

are somewhat lower than previously reported, largely due to the higher per cent of fat left in the present samples. Thiamine values previously reported range from 9.1 to 19.0 μg. per gram for fresh ham and 11.1 to 18.7 for fresh loins as compared to 7.7 to 14.8 for fresh hams and 7.4 to 15.2 for fresh loins used in this work. Nicotinic acid values previously reported range from 66 to 84 μg. per gram for fresh hams and from 60 to 67 for fresh loins as compared to 31 to 38 for fresh hams and 31 to 49 for fresh loins. Riboflavin values for fresh loins and hams are about the same as previously reported. This may be due to better extraction procedures used in the present determinations.

Pork hams appear to be higher in riboflavin and lower in nicotinic acid than pork loins. The thiamine content in micrograms per gram is about the same in fresh loins and hams, however. Different pork carcasses seem to vary considerably in vitamin content, even when the cuts are trimmed similarly. Thiamine values range from 7.4 to 15.2 μg. per gram fresh loin, and riboflavin values range from 1.6 to 3.0 μg. per gram. The nicotinic acid values are more constant, ranging from 31 to 49 μg. per gram of the fresh tissue, most of the samples being about 35 μg. per gram.

The vitamin content varies somewhat on different cuts from the same loins and hams, as can be seen from table 3. Sample numbers from 202–208, 210–219, 220–229, and 230–239 are from four different hogs. On fresh loin cuts from the series 210–219, for example, thiamine determinations are 13.4, 12.8 and 14.0 μg. per gram and values for fresh ham are 14.1 and 14.8 μg. per gram. This indicates variation in the thiamine content in different cuts from the same loins and hams. Nicotinic acid values for the same series are 37, 38, and 39 for fresh loin cuts and 31 and 31 μg. per gram of fresh ham. Riboflavin values are 2.6, 2.7, and 2.9 μg. per gram of fresh loin and 3.3 and 3.3 for fresh ham. This variation may be due to differences in the type of muscle in different sections of the loins and hams.

The riboflavin figures are higher in the cooked samples than in the fresh samples expressed on the micrograms per gram basis. This is due largely to the loss of water and fat during cooking and the high retention of riboflavin in the cooked meats. Thiamine and nicotinic acid values vary considerably in the cooked samples, depending on the method of cooking and the amount of fat and water lost during the process of cooking.

There is very little difference in total vitamin retention in braising, roasting and broiling. However, there is a difference in the amount of vitamin retained in the meat alone. Approximately 50% of the thiamine is retained in braised loin samples and 65–75% retained in other cooked samples. Similarly about 65% of the nicotinic acid is retained in braised loin samples and 75–90% retained by other cooked samples. Riboflavin retention after braising is about 75–80%, and from 80–100% is retained by other cooking procedures.

Both the method of cooking and size of the cut have an effect on the per cent retention in the meat, and a corresponding effect on the amount of each vitamin in the drippings. A large percentage of the vitamins is found in the drippings from braised loin cuts. This may be due to the larger surface area exposed, and the extracting effect of the condensing steam in the pan. The broiling of similar-sized cuts tends to minimize the per cent of vitamins present in the drippings. The small per cent of vitamins in the drippings from roast loins and hams is due largely to the size of the roast and the small surface area exposed.

As much as 24% of the original amount of thiamine is found in the drippings from braised loin samples, and about 6% is found in the drippings from broiled loin and ham cuts. Only 3–4% of the thiamine is found in the drippings from roast loins and hams.

A large proportion of the nicotinic acid is found in the drippings. As much as 37% is recovered from the drippings

after braising, and 5–15% of the nicotinic acid is found in the drippings from other cooked samples. A smaller percentage of riboflavin is found in the drippings, approximately 15% on braised loin drippings and 5–10% from other cooked samples.

SUMMARY

1. Thiamine, nicotinic acid, and riboflavin contents of pork hams and loins before and after cooking were determined.

2. The average retention in the meat alone is 70% for thiamine after roasting and broiling, and 50% after braising; 85% for nicotinic acid after roasting and broiling, and 65% after braising; and 85% of riboflavin as a result of any of the cooking methods.

3. The total retention in the meat plus the drippings is about the same for all the methods, an average of 70% for thiamine and at least 90% for riboflavin and nicotinic acid.

4. Appreciable amounts of each of the vitamins are found in the drippings, particularly from braised loin cuts.

5. A wide variation occurs in the thiamine and riboflavin contents of different pork carcasses, nicotinic acid being more constant.

The authors wish to express their thanks to Mr. James M. Price for technical assistance in carrying out the assays, Dr. Catherine J. Personius, Professor of Home Economics, for cooking the meats, and Mr. Robert W. Bray, of the Animal Husbandry Department, for selecting and cutting the meats.

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EXPERIMENTS UPON THE SIGNIFICANCE OF LIBERAL LEVELS OF INTAKE OF RIBOFLAVIN

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(Received for publication October 9, 1942)

Experiments have now been completed in which levels of riboflavin of from 1 to 10 μg. per gram of air-dry diet have been studied with respect to their effects upon different aspects of nutritional well-being and in many cases through at least two generations of the experimental animals (rats).

In the earlier series of these experiments there was found a difference between the minimal level of intake that would support growth, health and reproduction and the level which showed best results in long-term or two-generation experiments (Sherman and Ellis, '34). In the experiments recorded in the present paper, stepwise enrichments of the diet with riboflavin have been extended from the maximum of the first series to higher levels of intake; and the effects have been studied by means of a wider range of criteria of nutritional status.

EXPERIMENTAL

Each of our series of experiments was begun with matched lots of rats, taken from our laboratory colony and known to be of like hereditary and nutritional background. Such animals at the uniform initial age of 28 days were so distributed to the diets which were being compared in any one series as to afford a maximum of littermate control. The

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experimental observations included, either in all cases or in representative series: (1) body weight of each animal at the beginning of the experiment and at definite subsequent ages; (2) reproduction records of the females, including duration of reproductive life, and numbers of young born and reared; (3) concentration of hemoglobin in the blood of adults and of young; (4) length of life; (5) average weight of offspring at a standard age of 28 days, conventionally considered "end of infancy" in the rats of our colony; (6) gain in weight of the young when continued on the family diet; (7) gain in weight by representative young when transferred at 28 days of age to a riboflavin-deficient diet; and (8) growth and survival of representative young transferred when 28 days of age to a thiamine-deficient diet.

In the series of experiments with which this paper chiefly deals, the basal diet (designated as diet I in the tables) had the following percentage composition: ground whole wheat enriched with an alcoholic extract of 840 gm. whole wheat, 55.6; skim milk powder, 16.0; lactose, 8.0; casein (purified),[2] 6.0; butterfat, 8.0; cod liver oil, 2.0; Osborne and Mendel salt mixture, 2.0; agar agar, 2.0; and calcium phosphate, sec., 0.4.

Diets II and III consisted of the above diet I plus sufficient additions of essentially pure riboflavin[3] to bring their respective riboflavin values to about two and three times that of the basal diet as measured by the rat growth method in comparison with crystalline riboflavin. Subject to slight variations in the potencies of materials used, and to our errors of measurement, diets I, II and III contained respectively 3, 6.5 and 10 μg. of riboflavin per gram of the air-dry food mixture; or 0.9, 2.0, and 3.0 μg. of riboflavin per kilogram-calorie of food, respectively.

These three diets were fed under strictly parallel conditions to "lots" or "experimental families" each consisting of three females and two males taken as above described from our laboratory colony and so grouped that each animal on any

[2] Labco.

[3] Borden's 1E Lactoflavin.

TABLE 1

Age-weight data: Rats fed at three levels of riboflavin intake.

AGE IN DAYS	MALES						FEMALES					
	On diet I, 3 μg./gm.		On diet II, 6.5 μg./gm.		On diet III, 10 μg./gm.		On diet I, 3 μg./gm.		On diet II, 6.5 μg./gm.		On diet III, 10 μg./gm.	
	No. of cases	Wt.	No. of cases	Wt.	No. of cases	Wt.	No. of cases	Wt.	No. of cases	Wt.	No. of cases	Wt.
		gm.		gm.		gm.		gm.		gm.		gm.
28	32	45	32	46	32	46	48	44	48	44	48	45
40	32	76	32	77	32	77	48	70	48	72	48	71
60	32	139	32	140	32	142	48	115	48	116	48	118
90	32	211	32	207	32	213	48	162	48	164	48	164
120	32	252	32	248	32	257	48	172	48	178	48	180
150	32	284	32	277	32	288	48	193	48	192	48	197
180	32	299	32	292	32	305	48	201	48	198	48	200
210	32	308	32	305	32	319	48	206	47	206	48	211
240	32	315	32	311	32	326	48	211	47	213	47	214
270	32	318	31	317	32	334	48	215	47	214	46	223
300	31	324	31	315	32	336	48	216	47	215	46	222
365	31	319	30	307	31	328	48	220	47	217	46	229
400	31	317	28	306	31	323	48	218	46	220	46	231
500	31	293	23	295	29	302	44	226	45	217	43	228
600	26	279	19	277	23	289	43	221	38	217	38	223
700	14	270	8	288	15	266	33	211	31	208	32	214

TABLE 2

Adult vitality and length of life: Rats fed at three levels of riboflavin intake.

	ON DIET I		ON DIET II		ON DIET III	
	No. of cases	Mean ± P.E.[1]	No. of cases	Mean ± P.E.[1]	No. of cases	Mean ± P.E.[1]
Age of females at birth of first young (days)	45	117 ± 2.5	43	133 ± 5.2	46	120 ± 2.6
Total number of young born per female	48	29.0 ± 1.6	48	21.5 ± 1.1	48	24.3 ± 1.5
Total number of young reared per female	48	15.5 ± 1.1	48	12.4 ± 0.8	48	15.6 ± 1.2
Reproductive life (days)	48	251 ± 15	48	201 ± 11	48	228 ± 14
Length of life						
Males (days)	32	673 ± 13	32	616 ± 18	32	671 ± 14
Females (days)	48	758 ± 13	48	756 ± 16	48	729 ± 16

[1] Probable error of the mean.

one diet was matched by a litter mate of the same sex and essentially the same initial weight in the lot assigned to each of the other diets. Second-generation lots consisted of similarly parallel representative offspring of families on the respective diets. In tables 1 and 2, data for original experimental animals and for their offspring are averaged together.

Other representative offspring were used for other quantitative tests of nutritional status, as listed in the outline above and recorded in tables 3 and 4.

TABLE 3

Hemoglobin in blood of rats fed at three levels of riboflavin intake.

DESCRIPTION OF RATS	DIET I		DIET II		DIET III	
	No.	*Mean*	*No.*	*Mean*	*No.*	*Mean*
Males — 28 days, av. wt.	24	47	24	44	29	48
gm. hemoglobin per 100 cc.		12.5		12.2		12.5
Females — 28 days, av. wt.	20	47	22	43	23	45
gm. hemoglobin per 100 cc.		12.4		12.5		12.8
Males — 1 year, av. wt.	10	334	10	303	10	331
gm. hemoglobin per 100 cc.		15.4		15.4		15.4
Females — 1 year, av. wt.	10	223	11	224	10	236
gm. hemoglobin per 100 cc.		14.9		14.8		14.9

DISCUSSION

Diets I, II and III, equally good in other respects and containing respectively 3, 6.5, and 10 μg. of riboflavin per gram, all supported growth rates and body weights well within the normal range for animals of our colony.[4] The animals on the highest level of riboflavin intake will be seen from table 1 to have averaged slightly higher body weight at most ages than those on the lowest of the levels here studied. Such a difference would be logically consistent with some of our other observations as will appear below; but considered separately and objectively the difference does not appear statistically significant. That the animals on the intermediate

[4] As the custom of our laboratory has been to breed from rats of average size, our rats now may seem small compared with those of colonies in which the custom has been to "breed from the biggest and best."

TABLE 4

Comparison of records of offspring of families fed at three levels of riboflavin intake.

OBSERVATION	FROM DIET I			FROM DIET II			FROM DIET III		
	No. cases	Average initial weight	Mean ± P.E.[1]	No. cases	Average initial weight	Mean ± P.E.[1]	No. cases	Average initial weight	Mean ± P.E.[1]
Average weight of young at 28 days:									
Males (gm.)	407		43.9 ± 0.08	333		42.6 ± 0.1	453		43.7 ± 0.08
Females (gm.)	389		41.8 ± 0.08	328		42.3 ± 0.1	456		43.2 ± 0.08
Gain in weight 28–56 days on maternal diet:									
Males (gm.)	17	42	88.5 ± 2.4	23	51	84.5 ± 2.4	22	46	97.1 ± 1.6
Females (gm.)	19	43	64.8 ± 1.2	21	49	66.1 ± 1.6	23	48	73.4 ± 1.4
Gain in weight at 3 weeks on riboflavin deficient diet:									
Males (gm.)	19	55	4.6 ± 0.8	18	57	8.8 ± 0.4	19	53	10.4 ± 0.8
Females (gm.)	18	53	4.3 ± 0.7	18	55	6.5 ± 0.7	18	49	9.7 ± 0.7
Days of survival on thiamine-free diet:									
Males	8	44	33.1 ± 0.7	8	49	37.3 ± 0.7	8	50	38.9 ± 0.8
Females	7	42	35.6 ± 1.0	7	42	40.0 ± 1.3	7	48	44.6 ± 1.3

[1] Probable error of the mean.

level yield data very slightly below those on either of the other levels is presumably only an accident of normal physiological variability.

The age of the females at the birth of their first young (when, as in these experiments, the sexes grow up together), the number of young reared per female, and the duration of reproductive life are objective criteria of adult vitality. These data and the average lengths of life of the males and females separately are shown in table 2. In none of these respects did the animals on either of the higher levels of intake show superiority to those receiving diet I, i.e., at a level of 3 μg. of riboflavin per gram of dry food, or 0.9 μg. per kilogram calorie of the diet. Thus it appears that all the evidence supports the view that the diet I riboflavin level is on the "plateau" which supports optimal performance, at least in individuals living normally "protected" lives. It is interesting that this is essentially the same level of riboflavin relative to total food calories as is published by the National Research Council in its "Recommended Daily Allowances."

Furthermore, Van Duyne ('41) measured the riboflavin content of the liver, kidney, heart and skeletal muscle of adult rats from each of these three diets and found that the riboflavin content reached its plateau value at the level of intake of diet I (0.9 mg. of riboflavin per 1000 Calories).

Ellis and Bessey ('35) suggested riboflavin as one of the factors contributing to the maintenance of a high hemoglobin level, and György, Robscheit-Robbins and Whipple ('38) found the regeneration of hemoglobin to be hastened by dietary riboflavin. Hemoglobin determinations were therefore included in the experiments here reported, using the Newcomer method as described by Ellis and Bessey ('35); and here also it appears (table 3) that the riboflavin intake level of our present diet I (and of the "Recommended Daily Allowances" of the National Research Council) supports the full plateau value of hemoglobin concentration in the blood.

It is therefore of particular interest to find that with adult nutritional status so definitely plateaued at the intake level

of diet I, the threefold higher intake level of diet III did bring added benefit to the offspring. As shown by the data summarized in table 4, the young from families on diet III grew better when continued on the family diet and were better able to endure deprivation either of riboflavin or of thiamine than the corresponding young from diet I.

The last-mentioned and most noteworthy difference, originally reported by Ellis and Zmachinsky ('37) has been repeatedly confirmed in the course of the experiments here reported. Questions naturally arise as to the explanation of the beneficial effect, upon the young, of an increase above the intake level at which the adults appear to have reached their "plateau of optimal response" to liberality of riboflavin feeding. Whether the young have a relatively greater capacity for body storage of riboflavin than the adults, and whether the adults can store more when their food is further enriched in phosphate and protein as well as in riboflavin, are questions now under investigation. Whether the riboflavin-thiamine relationship means a literal sparing of thiamine by surplus riboflavin, or that as the tissue content of thiamine enzyme diminishes its function may be partially performed by abundance of the corresponding riboflavin enzyme, are also questions warranting further investigation. Riboflavin, thiamine, and nicotinic acid are sufficiently similar in the phosphate-protein combinations which they form in the tissues to call for such a study from the viewpoint of structural chemistry; and some experimental indications of an in vivo relationship between riboflavin and nicotinic-acid-enzyme levels have been reported by Hirt and Wimmer ('39) and by Axelrod, Sober and Elvehjem ('40).

SUMMARY

Experiments with diets (I, II, and III), otherwise similar, which contained, respectively, 3, 6.5, and 10 μg. of riboflavin per gram of air-dry food mixture were extended through successive generations of laboratory rats. The first of these levels seemed amply to support adult vitality and length of

life at the "plateau" of optimal nutritional status with reference to this vitamin. The threefold higher riboflavin intake level of diet III did, however, appear to confer added benefits upon the offspring of these experimental families, as shown by their somewhat more favorable growth when continued on the family diet and by their greater ability to withstand deprivation either of riboflavin or of thiamine.

ACKNOWLEDGMENT

This work was carried on with the aid of grants from the Rockefeller Foundation of New York City and from The Carnegie Institution of Washington.

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REPRODUCTION AND LACTATION OF MICE ON HIGHLY PURIFIED DIETS[1]

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ONE FIGURE

(Received for publication September 8, 1942)

Progress in the separation and identification of numerous dietary essentials, especially the vitamins, has brought within the realm of possibility the raising of animals on diets containing only substances of known chemical composition. Several recent papers have reported growth of animals, and in some cases reproduction, on diets containing no source of the vitamin B group except pure compounds. Jukes ('40) found that rats given choline chloride, pantothenic acid, nicotinic acid, riboflavin, thiamine and pyridoxine as the only supply of the B vitamins, grew at about 60% of normal rate and finally reached a weight of about 80% of normal. A few of his animals also reproduced, but reproduction and survival were below average. Unna ('40) and later Unna, Richards and Sampson ('41) reported growth and reproduction in rats on a purified diet containing the same B factors as those used by Jukes. With these same members of the B group added to a purified diet, Henderson, McIntire, Waisman and Elvehjem ('42) raised four generations of rats. Growth on their diet was good but consistently below normal. Woolley ('41) found that mice grew well on a purified diet containing these same B factors further supplemented with β alanine and inositol.

[1] A report of this work was given before the Physiological Society of Philadelphia at its meeting on May 19, 1942 (Am. J. Med. Sci., vol. 204, p. 155, 1942).

Troescher-Elam and Evans ('41) likewise obtained growth of mice on a diet containing p-aminobenzoic acid in addition to the B factors used by Woolley, but the inclusion of either fresh liver or a liver concentrate increased the rate of growth of their animals. Martin ('42) has recently reported "seemingly normal nutrition" of rats on a diet containing thiamine, riboflavin, pyridoxine, nicotinic acid, pantothenic acid, choline, inositol and p-aminobenzoic acid as the only supply of the B vitamins. With the same purified compounds and rice polish filtrate factor II added to a synthetic diet, Rogers, McElroy and Cowgill ('42) obtained reproduction with one strain of mice but failure with another strain. In contrast to the observations of Unna, Richards and Sampson and of Henderson and associates, Sure ('41) was unable to rear young rats successfully on a diet in which the B factors were supplied as thiamine, riboflavin, pyridoxine, choline chloride, pantothenic acid and a factor "W" concentrate. Lactation was improved by the addition of p-aminobenzoic acid.

In none of the above experiments was any attempt made to supply the fat-soluble vitamins and essential fatty acids entirely as pure compounds. However, Richardson, Hogan, Long and Itschner ('41) have recently reported the results of giving to rats a diet in which the fat-soluble as well as the water-soluble factors were given in pure form. On this diet the animals grew at a rate below normal, but when lard and a concentrate of vitamins A and D were added the animals not only grew at a rate comparable to those on a stock diet but also reproduced.

Nutrition experiments which are carried through from one generation to another not only serve to give information on the needs of the known factors for fertility and lactation but may better serve to demonstrate the existence of unrecognized dietary essentials. Such experiments, if carried through several generations, would practically eliminate the possibility of body storage, and the increased demand for reproduction and lactation would tend to exaggerate any deficiency that might exist. A logical approach at this time would seem to be an attempt to raise animals through several generations on a

diet containing nothing except compounds of known chemical composition. If this would be possible each factor could then be eliminated, one at a time, and subsequently the requirements of each essential for fertility and lactation could be determined. Given below are the results of raising mice through several generations on a highly purified diet in which all ingredients except the protein and cellulose (roughage) were supplied as pure chemical compounds.

EXPERIMENTAL

The basal diet used was composed of the following constituents expressed in percentage by weight: purified fibrin 25, salt mixture no. 12 (Jones and Foster, '42) 4, regenerated cellulose [2] 4 and glucose [3] 67. Fibrin was chosen as the protein in preference to casein because the former contains a higher content of sulfur-containing amino acids and has a simpler composition. Fibrin in contrast to casein is not a conjugated protein and, hence, if highly purified, would supply little to the diet except amino acids. Commercial beef-blood fibrin, after grinding, was extracted by percolation in the cold with 95%, 80% and 50% ethyl alcohol. The extraction with each concentration was continued until the extract was nearly colorless. The alcohol was followed by several extractions with very dilute acetic acid (1 cc. of glacial acetic acid per 6 liters of water) and then with distilled water until the washings were approximately neutral. The fibrin was next dried and ground, and as a last step it was extracted three times with ether. The salt mixture was composed entirely of c.p. materials.

The following members of the vitamin B group in the amounts indicated were incorporated into 100 gm. of the basal diet: thiamine chloride [4] 1 mg., riboflavin [4] 1 mg., pyridoxine [4] 1 mg., calcium panthothenate [4] 6 mg., nicotinic acid 6 mg., inositol 6 mg., para-aminobenzoic acid 15 mg. and choline chloride [4] 60 mg. These substances in amounts sufficient to

[2] Furnished through the courtesy of Dr. C. M. McCay, Cornell University, Ithaca, N. Y.

[3] Cerelose.

[4] Supplied by Merck and Company, Rahway, N. J.

last approximately 1 week were weighed on an analytical balance and all except the choline chloride were mixed together, ground in a mortar with a small portion of the diet and then mixed thoroughly with the proper amount of diet. Due to its hygroscopic nature the choline chloride was incorporated separately but in a similar manner.

As a source of the essential fatty acids a commercial linoleic acid [5] was used in the early part of these experiments. Although this product was designated as c.p. it undoubtedly contained some other fatty acids of quite similar composition, but was probably rather free of other contaminating materials. After these experiments had been in progress for 5 or 6 months it became impossible to obtain this preparation, and the unsaturated acids of linseed oil were prepared in the following way: The oil was saponified by boiling with alcoholic KOH, after which water was added and the solution of soaps extracted several times with ether to remove the non-saponifiable fraction. After the addition of H_2SO_4 to produce the free fatty acids, the latter were separated, washed several times with warm water and dissolved in boiling ethyl alcohol. Lead acetate in boiling alcohol was added in excess and the solution of lead soaps was allowed to stand overnight at 15° to 17°C. to allow the crystallization of the soaps of the saturated fatty acids. The alcoholic solution was decanted from the crystals and the alcohol driven off on a water-bath under reduced (water-pump) pressure. The unsaturated soaps were taken up in ether and the lead precipitated by the addition of H_2SO_4. The ether solution of the acids was washed with diluted H_2SO_4 until the washing gave no test for lead. The acids were then washed with water until the washings were neutral and the ether was driven off on a water-bath. In the following discussion this preparation is referred to as linoleic acid.

Crystalline vitamin A, crystalline vitamin D_2 (calciferol),[6] 2 methyl-1, 4-naphthoquinone (vitamin K), and alpha toco-

[5] Eimer and Amend.

[6] Supplied by Dr. O. W. Barlow of Winthrop Chemical Company, Inc., Rensselaer, N. Y.

pherol [7] were used as the fat-soluble vitamins and were fed at levels of 100 μg., 2.5 μg., 5 μg. and 6 mg. per 100 gm. of diet, respectively. Great care was taken to prevent the oxidation of these vitamins and the linoleic acid. A stock solution of vitamins D and K was made up together using linoleic acid as the solvent in a concentration such that 1 cc. would furnish enough of these vitamins to last all of the animals about 1 week. Separate stock solutions of vitamin A and tocopherol were prepared in a similar manner. These solutions were kept in glass-stoppered brown bottles in an atmosphere of CO_2 and stored in a refrigerator. As frequently as necessary, 1 cc. of each of the stock solutions was mixed with 13 cc. of linoleic acid. Each day this dilute solution was mixed with the diet in the proportion of 2 cc. per 100 gm. This dilute solution was also kept in a stoppered brown bottle and stored in the refrigerator. Each day after taking out the desired amount of solution, the bottle was swept out with a current of CO_2. By using the solutions of different concentrations as described, the necessity of exposing the entire solution to air each day was eliminated, but at the same time it was possible to prepare solutions of the vitamins in quantities which could be weighed fairly accurately. The vitamin D was weighed on a microbalance. There is no source of biotin in this diet, but biotin seems so widely distributed and so firmly combined with other substances such as proteins that so far it has been impossible to demonstrate the need for biotin except in diets containing a rather large percentage of raw egg white.

For the first 10 months of these experiments, or until the mice were in the third filial (F_3) generation, the animals were fed twice daily, the food residue discarded each time, and clean feeding cups provided. During the latter part of the experiment the animals were fed and given clean food cups but once daily.

Mice at about 140 days of age, at the time of their first mating, were put on this diet. Throughout these experiments one male was mated with two or three females. The results

[7] See footnote 4, page 163.

are shown in table 1. To afford a basis of comparison, the breeding results of mating seventy-one comparable females from our regular stock are given first (section A). These animals were part of the colony during the time the experiment was in progress. As seen, there were sixty-nine litters born

TABLE 1

Comparative breeding efficiency on stock diet, highly purified diet (diet 504), and purified diet plus liver extract diet (diet 508).

GENERATION OR MATING	NO. OF FEMALES MATED	NO. OF LITTERS BORN	PER CENT FERTILITY	NO. OF YOUNG BORN	AV. SIZE OF LITTERS	NO. OF YOUNG WEANED	PER CENT WEANED
A. Stock Diet							
	71	69	97	512	7.4	440	85
B. Four Successive Generations on Diet 504							
Parent	11	10	91	72	7.2	48	67 [1]
F_1 Generation	12	10	83	56	5.6	36	64 [1]
F_2 Generation	13	10	77	68	6.8	40	59 [1]
F_3 Generation	2	2	100	13	6.5	2	15 [1]
C. Three Successive Matings in F_1 Generation on Diet 504							
1st Mating	12	10	83	56	5.6	36	64 [1]
2nd Mating	12	12	100	89	7.4	54	61 [1]
3rd Mating	12	10	83	80	8.0	42	53 [1]
D. All Generations and Matings on Diet 504							
	73	63	86	447	7.1	272	61 [1]
E. Two Successive Generations on Diet 508							
Parent	7	7	100	31	4.4	17	55 [1]
F_1 Generation	5	3	60	17	5.7	9	53 [1]

[1] Indicates a significant difference between these data and corresponding data obtained with stock animals.

from seventy-one females mated, or 97% fertility. There was a total of 512 young, or an average of 7.4 per litter. Of these 512 young, 440, or 85%, were weaned.

In section B of table 1 are given the results of the matings of four successive generations on the highly purified diet (no. 504). Of the eleven original females, ten produced litters, giving a total of seventy-two young, of which forty-eight, or

67%, were weaned. Of these forty-eight young of the F_1 generation, twelve females were mated. Ten litters were born giving a total of fifty-six young, of which thirty-six, or 64%, were weaned. Again there were thirteen matings of the F_2 females; ten litters were born with a total of sixty-eight young, of which forty, or 59%, were weaned. One litter of this generation was born considerably earlier than the others, so two females of this litter were mated. Both of them reproduced, giving birth to thirteen young, of which only two were weaned.

These data were analyzed by the Chi square method and compared with similar data obtained with the stock animals. Although there were minor individual differences, there was no significant difference between the stock and the highly purified diets with respect to fertility, that is, the number of litters born compared with the number of females mated; also there was no significant difference with respect to size of litters. There was, however, a highly significant difference between the two diets with respect to the relative number of animals weaned. This might conceivably be interpreted to indicate that the outstanding deficiency of the highly purified diet was concerned with lactation or with the ability of the females to feed their young adequately. This will be discussed more fully later in the paper.

It sometimes happens that a given diet will support reproduction through more than one generation but that failure occurs when the same females are mated more than once. In view of this fact, the females of the F_1 generation were carried through several matings. The results are shown in section C of table 1. As seen, there is no difference from one mating to the next. Again the only statistically significant difference between the purified diet and the stock diet is in the percentage of survival to weaning.

In section D of table 1 are given the totals of females of all generations: mated, seventy-three; litters born, sixty-three; total young born, 447; average size of litter, 7.1; and average survival at weaning, 61%.

In figure 1 is shown the average growth of the animals on the purified diet as compared to those on the stock diet. The young were first weighed at 7 days of age. Separate curves are given for males and females. The dotted line represents the average growth of animals on the stock diet. The solid line is the average growth curve of the animals on the purified diet. Throughout the early part of life, the animals on the purified diet were smaller than those on the stock diet. This difference

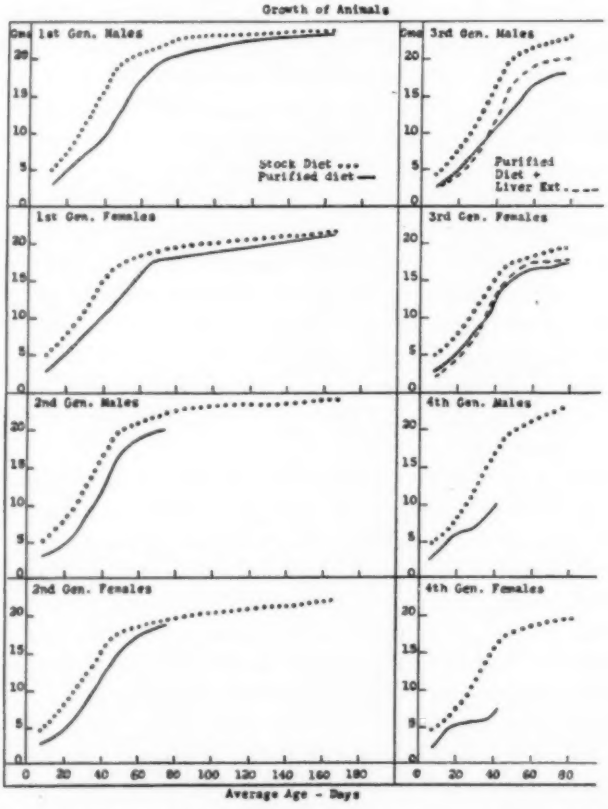

Fig. 1 A comparison of growth of mice on stock diet, purified diet (diet 504) and purified diet + liver extract (diet 508).

is significant until after weaning, when the surviving animals on the purified diet gradually overtook the controls.

Various workers have reported that the addition of liver extract to a diet in which the B factors were otherwise supplied as pure compounds increased the rate of growth of their animals. With these experiments in mind, several of the females of the F_2 generation were put on a diet identical with the highly purified diet 504 but to which had been added 2% liver extract E.[8] The addition of the liver extract had little effect on either survival or growth during the preweaning period, as shown by section E, table 1, and the growth curves indicated by the broken line in figure 1.

In the above experiments the young of the first litters of the parent and F_1 generations on the highly purified diet (diet 504) were weaned at 25 days of age. All others were weaned at 21 days except the young of the second mating of the F_1 animals on diet 504, which were weaned on the twenty-eighth day, and the young of the only matings of the F_1 animals on the purified diet plus liver extract (diet 508), which were weaned on the thirty-first day. In the latter case the young were so small that it was thought inadvisable to wean them at an earlier date.

The deficiency of the highly purified diet (diet 504), as judged by survival or growth, appears to be most pronounced during the preweaning period. At first thought this might be interpreted as indicating that this diet is deficient in some factor necessary for lactation but not for growth and development, and that an inadequate supply of milk is the cause of high mortality and poor growth during the preweaning period.

It is, however, possible that the deficient factor is necessary for growth and development and that it can be formed in the intestinal tract by microorganisms. During the preweaning period the normal flora has not been established and consequently an insufficient amount of the essential factor is produced. As the young animal becomes older, and especially after it begins to eat the regular diet, the flora becomes estab-

[8] Furnished by Dr. C. E. Graham of The Wilson Laboratories, Chicago, Ill.

lished and the factor is produced. If this is the case, it must be assumed that the microorganisms can produce a sufficient amount of the dietary essential to cover the animal's own needs but not enough to supply a litter of young through the milk.

We have made no attempt to measure the amount of milk secreted to determine which of these possibilities is the true explanation.

SUMMARY

Mice have been raised to the fourth generation of offspring on a diet in which all of the vitamins, the essential fatty acids and salts were supplied as chemically pure compounds, the rest of the diet being of highly purified materials. Fertility, as measured by the number of litters born and size of litters, was equal to that on a good stock diet, but growth during the preweaning period was subnormal and the mortality rate during the same period was higher than that of the stock animals. This would indicate that the diet used was not adequate.

Nothing can be said in regard to the particular type of deficiency with which we are dealing. It may be (1) an unknown substance, (2) an inadequate amount of one or more of the known dietary essentials, or (3) an imbalance of these known essentials. It does appear that 2% of liver extract will not completely correct the inadequacy of the diet.

The authors are indebted to Dr. J. Harold Austin for advice in the statistical treatment of the data, and to Miss Mabel E. Quinby and Miss Elise Weaver for assistance in the preparation of the diets and care of the animals.

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THE EFFECT OF PROTEIN AND B-VITAMIN LEVELS OF THE DIET UPON THE TISSUE CONTENT AND BALANCE OF RIBOFLAVIN AND NICOTINIC ACID IN RATS[1]

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(Received for publication August 14, 1942)

In a previous paper (Sarett, Klein and Perlzweig, '42) it was shown that the urinary excretion of riboflavin and nicotinic acid by dogs and of riboflavin by rats bore an inverse relationship to the level of protein intake. Riboflavin and nicotinic acid were inferred to be concerned with protein anabolism. Other workers had shown that an increase in the protein content of the diet required a corresponding increase in B-complex supplement for good growth of rats (Hartwell, '25; Reader and Drummond, '25). Similarly, retention of protein by chicks (Kleiber and Jukes, '42) and utilization of food for synthesis of tissue fat and protein (Sure and Dichek, '41) were both shown to require riboflavin.

In the present work, the effect of protein and B-vitamin levels of the diet upon nitrogen, riboflavin, nicotinic acid, thiamine and fat content of tissues was studied in rats by complete balance experiments.

EXPERIMENTAL

A group of twenty-nine young male rats (age 25 days), weighing 42 to 58 gm. (49 gm. average) were kept in metabolism cages in groups of four to six and fed the purified diets shown in table 1.

[1] Aided in part by the John and Mary R. Markle Foundation, the International Health Division of the Rockefeller Foundation and the Duke University Research Council.

The method of cooking these diets has been previously described (Sarett, Klein and Perlzweig, '42). The preliminary diet containing 18% casein was fed to all of the rats for 4 days. After a 24-hour fast, five animals were sacrificed for analysis. The remaining animals were divided into four groups of six each and fed the four experimental diets which were designed to provide two levels of protein intake (40 and 8%, respectively) and two levels of vitamin B complex intake (table 1).

TABLE 1

Composition of diets of varying protein and vitamin content.

	PRELIMINARY DIET	HIGH PROTEIN-HIGH VITAMIN (HPHV)	HIGH PROTEIN-LOW VITAMIN (HPLV)	LOW PROTEIN-HIGH VITAMIN (LPHV)	LOW PROTEIN-LOW VITAMIN (LPLV)
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Purified casein (Labco)	18	40	40	8	8
Agar	2	2	2	2	2
Corn starch	40	20	20	21	21
Sucrose	25	23	23	40	40
Beef suet	8	8	8	14	14
Cod liver oil	4	4	4	4	4
B. D. H. salt mixture [1]	4	4	4	4	4
Choline	0.1	0.1	0.1	0.1	0.1
Vitamin B complex [2]	One-half capsule	One-half capsule	One-fifth capsule	One-half capsule	One-fifth capsule
Water added to give 500 gm. of cooked diet					

[1] Prepared according to Drummond and Watson ('22).

[2] These capsules were generously supplied by Lederle Laboratories, Pearl River, New York. Each capsule contained the extract of 10 gm. of fresh liver, plus synthetic vitamins to give the following vitamin content: thiamine, 1 mg.; riboflavin, 1 mg.; nicotinic acid, 3.3 mg.; pyridoxine, 0.1 mg., and calcium pantothenate, 2 mg. The thiamine, riboflavin and nicotinic acid contents were checked by our own analyses.

The diets are designated high protein-high vitamin (HPHV), high protein-low vitamin (HPLV), low protein-high vitamin (LPHV) and low protein-low vitamin (LPLV). The diets low in B-vitamins permitted slow growth in the rat. The high vitamin B diets contained two and a half times the concentrations of the entire B complex. The diets were fed ad libitum.

The average daily food intake and the gain in weight for the rats in each group during the experimental period are given in table 2. After 12 days on these diets, the animals were again fasted 24 hours and two of each group sacrificed. The bodies of the remaining four rats in each group were analyzed under similar conditions on the twenty-seventh day.

TABLE 2

Food intake and weight gain per rat per day of rats on the four experimental diets.

DIET	WEIGHT GAIN	CALORIC INTAKE	PROTEIN INTAKE	RIBOFLAVIN INTAKE	THIAMINE INTAKE	NICOTINIC ACID INTAKE	CHOLINE INTAKE
	gm.	*cal.*	*gm.*	*µg.*	*µg.*	*µg.*	*mg.*
HPHV	3.2	28	2.4	33	33	110	6.5
HPLV	1.2	19	1.65	9	9	30	4.3
LPHV	1.2	28	0.52	33	33	110	6.5
LPLV	0.5	23	0.42	11	11	36	5.2

Urine was collected daily throughout the experiment and analyzed for riboflavin, nicotinic acid, thiamine and nitrogen by the methods previously used (Sarett, Klein and Perlzweig, '42). Feces were collected daily in acid and combined for the entire period for analysis. Nitrogen, riboflavin and nicotinic acid were determined on homogenized aliquots by the methods given for tissue in the next paragraph.

For carcass and liver analyses, the animals were killed by a blow on the head and the hair removed in BaS solution. The liver was removed for separate analysis, and the gastrointestinal tract discarded. The weighed carcass was run through a sausage grinder five to six times with thorough mixing, and a 20-gm. sample of the body of each rat was thoroughly homogenized in a Waring Blendor with enough water to give a final volume of 200 cc. The weighed liver was homogenized directly in the Blendor, with 49 cc. of water for each gram. Samples of the homogenized liver and carcass were immediately measured for each analysis. Nicotinic acid was determined chemically by the method of Dann and Handler ('41), nitrogen by the Kjeldahl method and the fat by alkaline digestion, acidification, extraction with ether and weighing.

For riboflavin analyses the samples were digested with 0.25 *N* H_2SO_4 for 1 hour in a water bath (Hodson and Norris, '39), neutralized to pH 5 to 6, made to volume and centrifuged after the precipitate began to settle. Filtration gave slightly lower values. An aliquot was treated with $KMnO_4$ and H_2O_2 (Ferrebee, '40) and the flavin determined fluorometrically. The microbiological method of Snell and Strong ('39) was used to check some of these riboflavin values; it agreed well with the fluorometric analyses on tissue as well as on urine and feces. Thiamine was determined by the yeast fermentation method (Atkin, Schultz and Frey, '39) on a portion of the neutralized digest prepared for the riboflavin analysis. The thiamine values obtained on this type of tissue extract are probably too high, since recovery experiments with several of the samples have given values of 120 to 170%. For this reason no thiamine balances are offered in this paper, but these relative values are useful for a comparison of tissue content.

Effect of B-vitamin and protein intake upon the vitamin and nitrogen content of the tissues. Table 3 gives the average composition of the livers and carcasses of each group of four rats analyzed after the 26-day period on the four experimental diets, HPHV, HPLV, LPHV and LPLV.

The analysis of the control group of five rats at the start of the experiment is included. The data obtained from analysis of two rats in each group on the twelfth day of this experiment, not shown in the table, substantiate the values given here for the twenty-seventh day.

The data for the carcasses show no difference in riboflavin content per gram in the four groups and show only slight differences in nicotinic acid concentration. Only the thiamine content per gram of tissue reflects the vitamin content of the diets.

Similarly, in the livers of these animals, the riboflavin and nicotinic acid concentrations are not correlated with the vitamin intake, but vary with the protein content of the diet. Even on the LPHV diet, the riboflavin and nicotinic acid concentrations in the liver appear to be limited by the amount of pro-

tein which is present. During the entire 26 days the group on the LPHV diet retained only 37 μg. of additional riboflavin in the carcass and none in the liver. The LPLV group retained no additional riboflavin in the carcass and actually lost some from the liver. The retention of additional nitrogen in both of these groups was very small, and none was retained in the livers of the LPLV group. The nicotinic acid balance is similar to that of riboflavin but may be less significant, since rats can synthesize nicotinic acid, especially with protein as

TABLE 3

Average vitamin and nitrogen content per rat of four groups after 26 days on diets varying in vitamin and protein content.

		WEIGHT	RIBOFLAVIN		NICOTINIC ACID		THIAMINE[1]		NITROGEN		FAT	
			Per gram	Total	Per gram	Total	Per gram	Total	Per gram	Total	Per cent	Total
		gm.	*μg.*	*μg.*	*μg.*	*mg.*	*μg.*	*μg.*	*mg.*	*gm.*		*gm.*
Controls (5 rats,	Carcass	51.5	3.9	200	47.2	2.45	3.0	155	26.6	1.37	7.8	4.02
60.5 gm.	Liver	2.8	27.2	75	153	.43	15	40	34.4	.095	4.4	.12
average)				275		2.88		195		1.47		4.14
27th day												
HPHV (4 rats,	Carcass	113	3.2	356	49.8	5.59	3.0	331	34.7	3.85	9.4	10.95
144 gm.	Liver	6.4	29.6	189	140	.87	15	95	34	.22	3.9	.24
average)				545		6.46		426		4.07		11.19
HPLV (4 rats,	Carcass	78	3.1	238	46.8	3.64	1.5	113	32.5	2.54	5.9	4.54
93 gm.	Liver	4.1	29.6	120	176	.72	9	37	35.1	.14	3.1	.13
average)				358		4.36		150		2.68		4.67
LPHV (4 rats,	Carcass	77.5	3.1	237	43.6	3.33	3.5	280	25.6	2.0	14.9	11.8
91.5 gm.	Liver	4.5	16.9	77	121	.55	21	99	27.1	.12	9.2	.42
average)				314		3.88		379		2.12		12.22
LPLV (4 rats,	Carcass	63	3.0	192	45.8	2.89	1.5	91	29.3	1.84	10.1	6.45
75 gm.	Liver	3.5	18.5	62	118	.40	8	27	27.2	.093	5.7	.20
average)				254		3.29		118		1.93		6.65

[1] Thiamine values are only approximate relative values. See text.

a precursor (Dann, '41; Huff and Perlzweig, '42). The unusually high nicotinic acid content of the livers of the HPLV group was not found in the analyses of this group on the twelfth day. The concentration and total content of thiamine in the liver parallel the vitamin content of the diet in the same fashion as do the carcass values. Both groups of animals on the low vitamin diets contained less thiamine at the end of the experiment than did the controls at the start.

The nitrogen content of both the liver and carcass is lower for the two groups of rats on the low protein diets, both per gram and per animal. Of the nitrogen ingested in the food, the percentages retained for growth by groups HPHV, HPLV, LPHV and LPLV were 25, 18, 31, and 28, respectively. The effect of the additional vitamin supplement in increasing the retention of protein was more marked in the high protein groups.

The fat content of the animals is higher per gram of tissue in the rats on the low protein diets than in those on the high protein diets. In each case, however, the group with the high vitamin intake contained more fat per gram and per animal, in both liver and carcass, than its corresponding group on the low vitamin intake.

Total balance of nitrogen, riboflavin and nicotinic acid of rats on the four experimental diets. From the tissue analyses, food content and the determination of urinary and fecal excretion, a balance has been calculated for nitrogen, riboflavin and nicotinic acid (table 4).

For riboflavin alone, the fecal content (analyzed fluorometrically and microbiologically) is of the same order of magnitude as the urinary excretion. If the fecal riboflavin is included in the balance, one finds a positive balance in the HPLV group, a negative balance in the LPHV group and neither an increase nor a loss in the other two groups. That this fecal riboflavin is mostly or entirely of bacterial origin is suggested by its relation to the fecal nitrogen and not to the vitamin intake, and by the unreasonable balances obtained if it is included. Coprophagy in vitamin B deficiency studies

TABLE 4

Average balance per rat of nitrogen, riboflavin and nicotinic acid of animals on the four experimental diets.

	DIET HPHV			DIET HPLV			DIET LPHV			DIET LPLV		
	Nitrogen	Riboflavin	Nicotinic acid	Nitrogen	Riboflavin	Nicotinic acid	Nitrogen	Riboflavin	Nicotinic acid	Nitrogen	Riboflavin	Nicotinic acid
	gm.	*μg.*	*mg.*	*gm.*	*μg.*	*mg.*	*gm.*	*μg.*	*mg.*	*gm.*	*μg.*	*mg.*
Content per control rat	1.46	275	2.83	1.46	275	2.83	1.46	275	2.83	1.46	275	2.83
Intake during 26 days	9.77	867	2.86	6.60	236	0.78	2.07	859	2.83	1.67	290	0.96
Total	11.23	1142	5.69	8.06	511	3.61	3.53	1134	5.66	3.13	565	3.79
Content per rat on 27th day	4.07	545	6.46	2.68	358	4.36	2.12	314	3.88	1.93	254	3.29
Urinary excretion	5.99	281	3.65 [1]	4.00	83	1.85 [1]	0.86	451	3.27 [1]	0.76	132	3.22 [1]
Fecal content	0.47	(300) [2]	(0.6) [2]	0.42	(200) [2]	(0.4) [2]	0.29	(180) [2]	(0.5) [2]	0.24	(160) [2]	(0.4) [2]
Total	10.53	826 [2]	10.11 [2]	7.10	441 [2]	6.21 [2]	3.27	765 [2]	7.15 [2]	2.93	386 [2]	6.51 [2]
Balance	—0.70	—316 [2]	+4.4 [2]	—0.96	—70 [2]	+2.6 [2]	—0.26	—369 [2]	+1.5 [2]	—0.20	—179 [2]	+2.7 [2]

[1] Includes total nicotinic acid and trigonelline of the urine.

[2] Fecal riboflavin and nicotinic acid are not included in the total and balance. See text.

has also given evidence of synthesis of the B vitamins in the intestine of the rat (Guerrant and Dutcher, '35). Further investigation is needed to determine how much of this riboflavin is available to the rat. Although the fecal nicotinic acid makes very little difference in this balance study, it has been shown that large doses of nicotinic acid in rats do not affect the fecal content (Huff and Perlzweig, unpublished data). Therefore, it is not included in the balance.

Excluding the fecal riboflavin from the balance, the data in table 4 for the groups HPHV, HPLV, LPHV and LPLV show that there remains unaccounted for in each rat 12, 3, 14 and 7 μg. of riboflavin per day, respectively. This seems to indicate an economy of utilization of riboflavin by the rats on the low vitamin diets, especially where the protein intake is high.

The data for nicotinic acid show that all four groups synthesized nicotinic acid, with the greatest amount synthesized in the HPHV group, which also showed the maximum growth. The synthesis of nicotinic acid by the rat has been demonstrated previously (Dann, '41; Huff and Perlzweig, '42).

The nitrogen balance lacks 0.7 and 0.96 gm. in the groups on the high protein diets and 0.26 and 0.20 gm. in the groups on the low protein diets. This represents 10 to 15% of the total nitrogen intake and gives an approximation of the validity of the other balance figures in table 4. These discrepancies are due partly to omission of the hair and intestine in the analyses, and to the small number of animals taken for the average control figure.

DISCUSSION

The preceding experiments show that the riboflavin and nicotinic acid contents per gram of rat carcass, excluding the liver, were the same for the four groups of rats maintained for 26 days on the diets varying in protein and B-vitamin content. Both the protein and vitamin contents of the diets limited the gain in weight of the animals, but the carcass tissue laid down

was similar in nicotinic acid and riboflavin concentration. This does not contradict the reports on the decrease in riboflavin content of the tissues of rats on diets deficient in riboflavin (Fraser, Topping and Isbell, '40; Axelrod, Sober and Elvehjem, '40), since the animals on the low vitamin intake in the present experiments each received a minimal intake of riboflavin, e.g., 9 and 11 μg. per day. The thiamine concentrations, however, in the carcass and liver of these same animals, who also received 9 and 11 μg. of thiamine per day, were only about one-half of that found in the rats receiving 33 μg. of thiamine per day. The protein level of the diet did not appear to influence the thiamine concentration in the tissues.

The riboflavin and nicotinic acid content per gram of liver was low in the animals on both low protein diets regardless of vitamin intake, and corresponded somewhat to the decreased nitrogen content. Conversely, on both high protein diets, regardless of vitamin intake, the riboflavin and nicotinic acid content per gram of liver was high. These low values for riboflavin and nicotinic acid content of liver on the low protein diets, with normal values for the remainder of the carcass, suggest that the carcass levels are maintained at the expense of the liver, which acts as a store for the excess riboflavin. For the retention of riboflavin and nicotinic acid in the liver, protein deposition appears to be necessary. This is in agreement with the previous findings (Sarett, Klein and Perlzweig, '42) that the urinary excretion of riboflavin and nicotinic acid by dogs and of riboflavin by rats was increased when there was little or no nitrogen retention. The experiments of Supplee, Jensen, Bender and Kahlenberg ('42) in which the low riboflavin levels in the livers of deficient rats were only transiently increased by doses of riboflavin also suggest that the carcass levels may be replenished before those of the liver.

The increases in fat content of liver and carcass, both per gram and per rat, resulting from increasing the B complex supplement of the diet agree with the evidence that some

of the B complex vitamins, riboflavin (Sure and Dichek, '41) and thiamine and pyridoxine (McHenry and Gavin, '41), are necessary for the synthesis or deposition of body fat. The increases in fat content resulting from the decrease of the protein content of the diet may have been due only to a lowering of the methionine intake, since each of the rats received only 5 to 6 mg. of choline per day.

The balance studies show less destruction of riboflavin by the rats on diets low in riboflavin. The riboflavin in the feces is considerable and is of the order of magnitude of that found in the urine. However, the fecal riboflavin appears to be of bacterial origin and may not be available to the rat.

SUMMARY

The concentrations of riboflavin and nicotinic acid found in the carcasses of rats grown for 26 days on the four experimental diets were approximately the same, and were not affected by either minimal and high levels of the B-vitamin complex or low and high protein content of the diet. In the liver the concentrations of riboflavin and nicotinic acid varied directly with the level of protein in the diet and were independent of the vitamin intake.

The concentrations of thiamine in the carcass and liver reflected the level of B-complex intake but were not affected by the level of protein in the diet.

The deposition of fat in the liver and body was augmented by either increasing the B-complex supplement or lowering the protein content of the diet; the maximal effect was obtained on the low protein-high vitamin diet.

Balance studies show a synthesis of nicotinic acid by all four groups of rats and an economy of utilization of riboflavin by rats on diets low in B-vitamins.

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RENAL THRESHOLD FOR ASCORBIC ACID IN TWELVE NORMAL ADULTS [1]

WITH A NOTE ON THE STATE OF TISSUE RESERVES OF SUBJECTS ON AN INTAKE OF ASCORBIC ACID APPROXIMATING THE SUGGESTED DAILY ALLOWANCE

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ONE FIGURE

(Received for publication September 30, 1942)

The renal threshold for ascorbic acid determines the concentration of ascorbic acid maintained in the plasma of an individual whose supply of vitamin C is liberal. A person whose renal threshold for ascorbic acid is high, maintains a given plasma concentration on a lower intake of ascorbic acid, and wastes less ascorbic acid than does one whose renal threshold is low.

Most estimations of the renal threshold for ascorbic acid have been based on a few determinations on each of a number of individuals. Such estimates represent the mean value for the group and give no indication of the extent to which individual values vary from the mean.

Several investigators have estimated the average renal threshold for ascorbic acid for fairly large groups of subjects.

[1] Most of the data in this paper were included in theses presented to the faculty of the Graduate School of Cornell University in fulfillment of the requirement for the Ph.D. degree, June, 1941, by Clara A. Storvick, and for the M.S. degree, May, 1942, by Jane D. Sanford. Two subjects were studied by Clara A. Storvick at the University of Washington during 1941–1942.

Faulkner and Taylor ('38) concluded from observations on one hundred and seventy-five individuals (fifty normal subjects and one hundred and twenty-five patients) that "ascorbic acid is a threshold substance with a critical level of excretion in the vicinity of 1.40 mgm. per 100 ml. serum." Goldsmith and Ellinger ('39), who made a scatter diagram from seventy-five observations on twenty-two subjects, noted that "little vitamin C was excreted by the kidneys until the level in the blood reached approximately 1.4 mg. per hundred cubic centimeters, at which time an increase in the excretion of vitamin C occurred." Friedman, Sherry and Ralli ('40) made sixty-nine observations on nineteen subjects whose plasma ascorbic acid concentrations ranged from 0.03 to 2.05 mg. per cent. They found that at plasma concentrations above 1.5 mg. per cent excretion of vitamin C increased. These estimates of renal threshold for ascorbic acid, based on group studies, are remarkably similar.

Other values, both lower and higher, may be found in the literature. Crandon and associates ('40), who estimated the renal threshold of their subject during recovery from experimental scurvy say, "According to our findings, however, this threshold does not occur at a plasma value of 1.4 mg. per 100 cc. . . . but rather with a plasma value of about 0.85 mg. in the normal adult. . . ." These authors suggest that in a totally vitamin C-deficient state this renal threshold may be lower. Falke ('39) estimated the "critical level" for his subject as 1.6 to 1.8 mg. per cent. Both Crandon and associates ('40) and Falke ('39) imply that the values which they observed in their subjects apply to normal adults in general. Abt and Farmer ('38), however, expressed their belief that the plasma level at which a marked urinary excretion occurs varies considerably with the individual.

In the present study, the renal threshold for ascorbic acid was estimated for twelve normal adults, four men and eight women. From thirty-three to ninety-three determinations were made on individual subjects, covering a range of values on either side of the threshold, so that the threshold value for

each was estimated with a reasonable degree of accuracy. The renal threshold values obtained for ten of the twelve subjects were within the relatively narrow range of 1.10 to 1.30 mg. per cent, but for the other two subjects, the higher thresholds, 1.45 and 1.80 mg. per cent, respectively, indicate that marked individual variations occur.

EXPERIMENTAL PROCEDURE

Data concerning the subjects used in the present study are given in table 1.

TABLE 1

Sex, age, height, and weight of the twelve subjects.

SUBJECT	SEX	AGE	HEIGHT	AV. WEIGHT
			inches	*pounds*
I. P.	F	26	62	121
W. T.	M	24	71½	163
J. M.	M	23	68	155
J. B.	F	29	62	129
M. S.	F	20	67	142
R. F.	M	26	65½	136
J. S.	F	22	66½	133
C. S.	F	34	68½	149
H. H.	F	40	62	131
H. L.	M	25	71	164
B. H.	F	21	67½	130
S. H.	F	23	64	130

The diet given to subjects C. S., H. H., B. H., and S. H., and in some of the experiments to H. L. and J. S., was the same as that reported previously (Belser, Hauck and Storvick, '39). For the remainder of the experiments, three changes were made in the diet to insure its adequacy in terms of the allowances proposed by the Committee on Food and Nutrition of the National Research Council ('41): green beans were substituted for beets; the evaporated milk intake was increased to 120 gm. per day; and each subject was required to eat at least 150 gm. of cooked cereal.[2] The ascorbic acid content of the modified diet, as estimated from indophenol titration (Bessey, '39), was approximately 14 mg.

[2] Made with one-half wheat germ and one-half Ralston's cereal.

The ascorbic acid supplement [3] varied in amount, depending on the primary purpose of the experiment. Details of some of the experiments have been published (Storvick and Hauck, '42).

The methods for the collection and preservation of urine and for the determination of ascorbic acid therein were the same as those reported previously (Belser, Hauck and Storvick, '39). Reduced ascorbic acid in plasma was determined by the micromethod of Farmer and Abt ('36).

In the first series of experiments, most of the determinations of plasma ascorbic acid content were made hourly for 6 hours after a test dose. Urine was collected at 1- or 2-hour intervals, the urine specimen being obtained just before the blood sample was taken.

In the second group of experiments, which was designed primarily for a study of the renal threshold for ascorbic acid, the blood sample was taken midway in the hour for which the urine specimen was collected, i.e., if the urine specimen was obtained for the hour from 8 to 9 A.M., the blood sample for this hour was obtained at 8:30 A.M. Also for this series, daily determinations were made of fasting plasma ascorbic acid content, and the ascorbic acid excretion for the corresponding hour. Hourly determinations of plasma and urinary ascorbic acid were also made for the 6 hours following ingestion of ascorbic acid on the first day of the experiment, on the sixth or seventh day, and at the end of the experimental period, when a test dose was given. Thus values below and above the renal threshold were obtained.

RESULTS AND DISCUSSION

Estimation of the renal threshold for ascorbic acid. Plasma ascorbic acid values for each subject were arrayed with the corresponding values for urinary excretion of ascorbic acid. Scatter diagrams were also made (fig. 1). The arrayed data and the scatter diagram for each subject were examined to see at what plasma value there appeared to be a marked rise in

[3] Hoffmann-La Roche.

urinary excretion. The means of the urinary values were then calculated for the plasma values near the apparent renal threshold and for all lower plasma ascorbic acid values. That is, if the renal threshold appeared to be approximately 1.30 mg. per cent, the mean was determined for the urinary values which corresponded to the plasma ascorbic acid concentrations between 1.25 and 1.34 mg. per cent. If values were too few

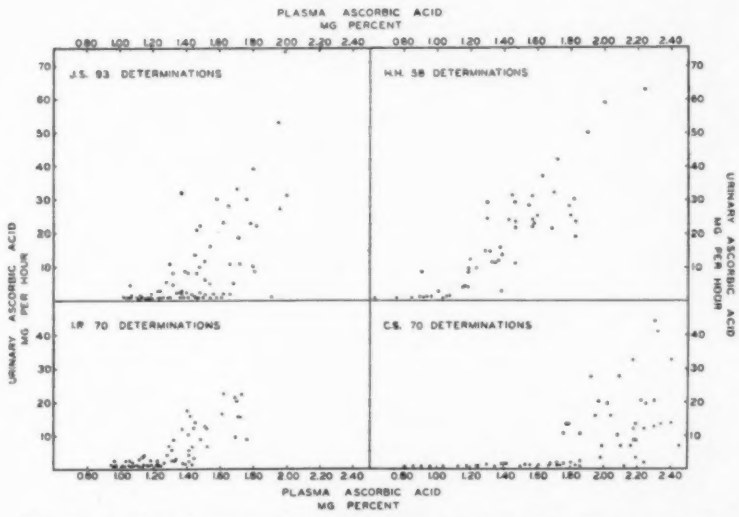

Fig. 1 Hourly urinary ascorbic acid values plotted against the corresponding values for plasma ascorbic acid, for four subjects.

between these limits, the mean was determined for those occurring between 1.20 and 1.39 mg. per cent. The differences between the means were submitted to Student's ('25) "t" test for determination of mathematical significance. In the same manner, mean plasma values above and below the apparent renal threshold were tested. The estimated renal thresholds for the twelve subjects, with the results of the statistical treatment, are shown in table 2. Note that in general the odds were high for the apparent renal threshold and for the plasma value above it, but the odds were low for the plasma

value below the apparent renal threshold. In general, the estimates of the renal threshold based on a larger number of values have greater statistical significance than have those based on a smaller number of values. The method appears to give a reasonable approximation of the true threshold values.

The estimated renal threshold values for these twelve subjects ranged from 1.1 to 1.8 mg. per cent. Ten of the subjects had threshold values between 1.1 and 1.3 mg. per cent.

TABLE 2

Estimated renal threshold for ascorbic acid for twelve subjects.

SUBJECT	TOTAL NUMBER OF DETERMINATIONS	STATISTICAL SIGNIFICANCE FOR VALUES TESTED					
		Apparent renal threshold		Plasma value above renal threshold		Plasma value below renal threshold	
		Mg. %	Odds [1]	Mg. %	Odds [1]	Mg. %	Odds [1]
I. P.	70	1.30	9999 : 1	1.40	9999 : 1	1.20	1 : 1
W. T.	38	1.10	49 : 1	1.30	9999 : 1	1.00	2 : 1
J. M.	42	1.10	9999 : 1	1.20	18 : 1	1.00	1 : 1
J. B.	35	1.30	9999 : 1	1.40	4999 : 1	1.20	43 : 1
M. S.	44	1.30	99 : 1	1.40	999 : 1	1.15	2 : 1
R. F.	40	1.10	99 : 1	1.20	99 : 1	1.00	19 : 1
J. S. [2]	93	1.30	9999 : 1	1.40	999 : 1	1.20	7 : 1
C. S. [2]	70	1.80	9999 : 1	1.90	9999 : 1	1.70	27 : 1
H. L. [2]	33	1.20	99 : 1	1.40	999 : 1	1.00	16 : 1
H. H. [2]	58	1.20	9999 : 1	1.30	9999 : 1	1.05	3 : 1
B. H. [2]	48	1.45	9999 : 1	1.60	9999 : 1	1.35	8 : 1
S. H. [2]	36	1.25	9999 : 1	1.45	9999 : 1	1.05	4 : 1

[1] Odds that the difference between the mean urinary excretion corresponding to this plasma ascorbic acid value, and the mean urinary excretion for all plasma values below the apparent renal threshold, is not due to chance.

[2] Data for subjects C. S., H. H., B. H., and S. H., and some data for subjects J. S. and H. L. were obtained by the first method described.

When data yielded by subjects J. S. and I. P. in two experimental periods were calculated separately, the same threshold values were obtained as when all data for each of these subjects were pooled. The same threshold value resulted for J. S. when representative plasma values were obtained by averaging the values for the beginning and end of the hour for which urine was collected, as when a year

later, in the second series of experiments, plasma ascorbic acid determinations were made midway of the hour representing the urine specimen.

In some studies recorded in the literature, the plasma ascorbic acid content corresponding to the first marked rise in urinary excretion was considered to be the renal threshold; in others, in which the threshold was suddenly exceeded by an intravenous dose of ascorbic acid, the plasma value at which a marked decrease in excretion occurred was so considered. The peak in the plasma ascorbic acid content following a test dose precedes the peak in urinary excretion; and when plasma ascorbic acid content decreases, it may reach or fall below the threshold in an hour during which an appreciable amount of ascorbic acid appears in the urine. If the plasma value at the first marked rise in urinary excretion of ascorbic acid is considered as the renal threshold, this value tends to be higher than the actual threshold. On the other hand, the estimated threshold may be somewhat lower than the actual threshold value if the point where the urinary excretion approaches zero is used. In the present studies, values were obtained during periods both when the plasma ascorbic acid was rising and when it was decreasing. This minimizes the effect of the lag in urinary excretion following a test dose on the apparent renal threshold.

When the plasma ascorbic acid level is maintained at or above the renal threshold, the subject is presumed to be saturated with respect to vitamin C (Faulkner and Taylor, '38). For nine of the twelve subjects used in this study data were available on the mean fasting plasma ascorbic acid values during tissue saturation (table 3). For five of the subjects, J. S., I. P., J. M., B. H., and S. H., the estimated renal thresholds were approximately the same as the mean fasting plasma values during tissue saturation; for three subjects, H. L., H. H., and C. S., the estimated threshold value was slightly higher, and for one, W. T., it was slightly lower than the mean fasting plasma value during tissue saturation. In view of the limitations of the method, and the daily variation in fasting

TABLE 3

Mean fasting plasma ascorbic acid,[1] renal threshold, urinary response to a 400-mg. test dose, and "utilization" values for subjects on various levels of ascorbic acid intake.

SUBJECT	ASCORBIC ACID INTAKE	LENGTH OF EXPERIMENT	MEAN FASTING PLASMA ASCORBIC ACID	RENAL THRESHOLD	URINARY RESPONSE TO TEST DOSE	"UTILIZATION"[2]
	mg.	*days*	*mg. %*	*mg. %*	*mg.*	*mg.*
I. P.	74	13	1.1	1.30	245	35
	74[3]	12	1.0		263	31
	214	13	1.2		285	41
	214	18	1.3		267	63
W. T.	74	13	1.0	1.10	134	41
	214	18	1.2		270	70
J. M.	74	14	1.0	1.10	74	45
	214	18	1.1		237	70
J. B.	74	14	1.1	1.30	223	30
M. S.	74	13	1.1	1.30	157	44
J. S.	74	13	1.2	1.30	246	40
	74[3]	12	1.2		239	41
	89	13	1.3		284	35
	210	28	1.3		268	43
C. S.	135	28	1.7	1.80	207	53
	210	28	1.6		261	56
H. L.	135	28	1.1	1.20	234	70
	210	28	1.1		261	75
H. H.	135	28	1.1	1.20	214	48
	210	28	1.1		267	47
B. H.	ad lib.[4]	28	1.5	1.45		
S. H.	ad lib.[4]	28	1.3	1.25		

[1] Values obtained on the first 3 days of the experiment were omitted in calculating the means.

[2] "Utilization" values were calculated by subtracting the mean daily urinary excretion of ascorbic acid from the ascorbic acid intake.

[3] Three 20-mg. supplements of ascorbic acid were given, one at each meal, instead of the usual single supplement at breakfast.

[4] The mean daily urinary excretion of ascorbic acid was high: for B. H., 84 ± 11 mg. and for S. H., 93 ± 7 mg. These subjects were probably in a state of tissue saturation.

plasma ascorbic acid in subjects on a controlled intake (Storvick and Hauck, '42), the agreement in the two sets of values may be considered to be reasonably close.

Since the renal thresholds for ascorbic acid in these twelve subjects ranged from 1.1 to 1.8 mg. per cent, it seems probable that even greater individual variations in renal threshold than those observed, may exist among normal individuals. For example, three of the ambulatory patients observed by Goldsmith, Ogaard and Gowe ('41) probably had higher renal thresholds than did the subjects of this study, since weekly determinations of the fasting plasma ascorbic acid values over periods of from 13 to 16 weeks on a partially controlled diet averaged 1.7, 1.9 and 2.1 mg. per cent.

The threshold value of 0.85 mg. per cent reported by Crandon et al. ('40) is lower than the lowest value observed in this study. This low threshold value may have been related to the previous deficiency of vitamin C in the subject, or this subject's renal threshold for ascorbic acid might be low under normal conditions.

Subjects such as W. T., J. M., and R. F., all with renal thresholds of 1.1 mg. per cent, cannot maintain a plasma value of 1.4 mg. per cent which, according to Faulkner and Taylor ('38), indicates tissue saturation, since as soon as the threshold value is reached the excess is excreted through the kidneys. On the other hand, a subject such as C. S. with a renal threshold of 1.8 mg. per cent can maintain a plasma value of 1.4 mg. per cent with a relatively small loss in the urine (Storvick and Hauck, '42). The optimum plasma ascorbic acid content for health is not known, but obviously a person with a high renal threshold can maintain a given plasma ascorbic acid level on a lower intake of ascorbic acid than can a person with a low renal threshold for this substance.

State of tissue reserves of ascorbic acid maintained on an intake approximating the suggested daily allowance for adults. In the second series of experiments, the intake of ascorbic acid, i.e., 60-mg. supplement plus about 14 mg. in food, approximated that recommended for adults by the Committee on Food

and Nutrition of the National Research Council ('41). This level of intake was chosen to obtain information on the state of tissue reserves of adults whose intakes of ascorbic acid approximated the suggested allowance.

Various criteria are used to judge whether or not an individual is saturated with vitamin C. Data used in estimating the state of tissue reserves in these subjects are included in table 3. If the excretion of half of the test dose within 24 hours is taken as the criterion for tissue saturation (Smith, '38), subjects J. S., I. P., and J. B. would be considered in a state of tissue saturation on a 74-mg. intake. All of the subjects excreted more than 50% of the 400-mg. test dose on the second day on which it was given; therefore, none of them had suffered much depletion of tissue reserves during the 12- to 14-day period on a 74-mg. daily intake of ascorbic acid.

The subject's own previous test dose responses following periods when the tissues are known to be saturated, may also be used as the basis for judging tissue saturation (Belser, Hauck and Storvick, '39). Subject I. P., following the period when the supplement was divided, excreted approximately as much ascorbic acid in response to the test dose as she did following one period in which the daily intake was 214 mg. None of the other subjects could be considered to be saturated on the basis of this criterion.

Individuals may be considered to be in a state of tissue saturation if the mean fasting plasma ascorbic acid value equals or exceeds the renal threshold (Faulkner and Taylor, '38). All six of these subjects, however, had mean fasting plasma ascorbic acid values on the 74-mg. intake which were slightly, but perhaps not significantly, lower than both the estimated renal threshold and mean fasting plasma ascorbic acid values observed on daily intakes of over 200 mg. of ascorbic acid.

"Utilization" values are generally higher for the saturated individual than for one whose intake of ascorbic acid is insufficient to maintain tissue saturation (Hathaway and Meyer, '41; Ralli, Friedman and Sherry, '39). Of these subjects,

J. S. "utilized" approximately the same amount of ascorbic acid on a 74-mg. intake as on an intake of over 200 mg.; I. P. "utilized" slightly more, and W. T. and J. M. distinctly more on the higher intake than on an intake of 74 mg.

When all available data are considered it appears that subjects J. S., I. P., and J. B. were saturated or nearly so, on a 74-mg. intake, but that this intake was insufficient to maintain tissue saturation in subjects W. T., J. M., and M. S. The data presented here, together with those previously published (Storvick and Hauck, '42) indicate that subject J. S. required between 74 mg. and 89 mg. of ascorbic acid daily to maintain tissue saturation. More data are needed to determine whether longer periods on the recommended daily allowance would result in marked depletion of tissue reserves in some subjects, or whether they would become adjusted to the level of intake, as was suggested by Van Eekelen ('36).

SUMMARY

Renal threshold values for ascorbic acid for twelve normal adults, four men and eight women, ranged from 1.1 to 1.8 mg. per cent. Values for ten of the twelve subjects were within a range of 1.1 to 1.3 mg. per cent.

Of six subjects receiving 74 mg. of ascorbic acid daily for from 12 to 14 days, three maintained tissue saturation if the excretion of half of the test dose in 24 hours is used as a criterion, whereas three gave evidence of slight depletion of tissue reserves of ascorbic acid on this intake.

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UTILIZATION OF UREA BY YOUNG CALVES

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ONE FIGURE

(Received for publication October 10, 1942)

It is accepted that during growth cattle and sheep are able to use a certain amount of urea in the synthesis of body proteins. The formation of protein is probably accomplished by bacterial action in the rumen. In past experiments, cattle (Fingerling et al., '37; Bartlett and Cotton, '38; Hart et al., '39) and sheep (Harris and Mitchell, '41) in which the rumen was already well developed were used. The present study was made to determine the value of urea for young calves during the period when the activity of the growing rumen was developing. It was also decided to study the effect of added supplements of the B vitamins in an effort to measure the possible influence of these factors upon the initiation of rumen function.

PLAN OF THE EXPERIMENT

Two male Holstein calves a few days of age were fed whole milk and a low-protein basal diet composed of yellow corn 20 parts, chopped timothy hay 30, corn starch 37, cane molasses 10, bone meal 2 and salt 1. Four other calves were fed a similar diet in which 4 parts urea (11.8% protein equivalent — $N \times 6.25$) replaced an equal weight of starch. After 4 weeks whole milk was fed in decreasing amounts so that at about 2 months of age the milk was omitted from the diet entirely and the calves were fed the experimental diet only. One-half of

the calves in each group received a daily supplement of the B vitamins as follows: 0.1 mg. per kilo body weight of thiamine, riboflavin, calcium pantothenate, pyridoxine and p-amino benzoic acid, and 1.0 mg. per kilo of nicotinic acid and choline. Vitamin A and D concentrate was fed twice weekly to supply 100 I.U. vitamin A and 10 I.U. vitamin D per kilo body weight per day. Alpha-tocopherol was fed at the rate of 2.0 mg. per kilo per day. The calves were fed individually, kept in individual pens about 4×8 feet, and bedded with sawdust. Water was allowed twice daily.

The calves were weighed once each week and measurements of the heart girth and height at withers were taken for comparison with calves fed normal diets. The calves were placed in metabolism stalls at 3 months of age, and after a 10-day preliminary period on a constant food intake the feces and urine were collected for a 10-day period. The excreta were studied to obtain digestion coefficients and nitrogen balance data for each ration.

RESULTS

The relative growth rates of the calves on the different diets are shown in figure 1. All of the calves increased in body weight at a substantially normal rate during the first 2 months

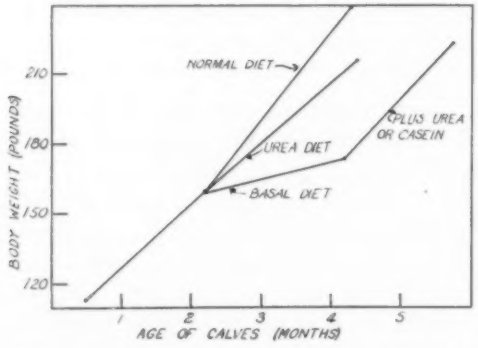

Fig. 1 The relative growth rates of calves fed the basal diet, the basal diet plus urea and a normal diet.

when whole milk was fed. On the low-protein basal diet the calves grew for about 2 weeks after milk was omitted and then remained at a constant weight. They gained an average of 7 pounds in 2 months. During this time they were in large negative nitrogen balance, as is described later. The calves fed the urea diet continued to grow at a fairly constant rate. They gained 61 pounds on the average during the 2 months as compared with a gain of 80 pounds for calves treated in a similar manner but fed a normal ration. At 4 months of age the urea-fed calves were 75 to 90% of the normal in weight (Morrison, '36). In height at withers and heart girth they were 95% of normal.

The calves on the basal ration were far below normal in height at withers and heart girth as well as in body weight, the hair coat was extremely rough, and the animals were unthrifty in appearance. When the calves on the basal diet were 4 months of age one of them was given urea and the other casein to increase the dietary protein equivalent to a level of 16.2% ($N \times 6.25$). The calves began to eat more feed and started to gain in weight immediately (fig. 1). They gained 50 pounds in the next 47 days. Supplements of the B vitamins had no apparent influence upon the growth rate or general thriftiness of the calves on either diet.

When the calves were 3 months of age they were placed in metabolism stalls. After a constant intake period of at least 10 days the feces and urine were collected for 10 days. From an inspection of the summary data for the digestion trial (table 1) it is clear that the calves on the basal diet digested their feed much less efficiently than did the animals which received the urea diet. The striking difference seen in the apparent digestibility of the dietary nitrogen can be explained, at least in part, by the presence of a relatively larger amount of metabolic nitrogen in the feces of the animals fed the nitrogen-low diet. The depressed digestibility of carbohydrates may be due to a reduction in the bacterial activity in the rumen.

TABLE 1

The apparent digestibility of the experimental rations.

CALF NO.	DIET	DRY MATTER INTAKE PER 100 LB. BODY WEIGHT	COEFFICIENTS OF APPARENT DIGESTIBILITY		
			Dry matter	Nitrogen	Carbohydrates
		gm./day	%	%	%
21	Urea plus B vitamins	986	74.4	77.5	75.0
22	Urea	907	74.4	77.6	75.2
23	Urea	840	77.1	81.1	77.2
24	Urea plus B vitamins	903	80.9	80.8	81.9
25	Basal	662	57.2	3.6	60.6
26	Basal plus B vitamins	708	60.2	30.3	63.0

TABLE 2

Nitrogen balance data for growing calves expressed on a daily basis.

CALF NO.	BODY WEIGHT	PROTEIN EQUIVALENT OF DIET	INTAKE	NITROGEN			RETAINED OF DIETARY N
				In feces	In urine	Balance	
	lb.	%	*gm.*	*gm.*	*gm.*	*gm.*	%
21	206	16.2	54.7	12.3	26.8	+15.6	28.6
22	224	16.2	54.7	12.3	24.5	+17.9	32.7
23	202	16.2	45.7	8.6	26.1	+11.0	24.1
24	190	16.2	45.7	8.7	20.5	+16.5	36.1
25	170	4.4	8.1	7.8	4.9	— 4.6	...
26	174	4.4	8.9	6.2	12.2	— 9.5	...

The nitrogen balance data are shown in table 2. The calves receiving urea were in positive nitrogen balance. They retained from 24 to 36% of the nitrogen in the diet. In striking contrast, the calves on the low protein basal diet were in negative balance. Using values for young cattle (Harris, '42) of 0.034 gm. of endogenous nitrogen per kilo of body weight and 4.89 gm. metabolic fecal nitrogen per kilo of dry matter intake the calculated biological values for the dietary nitrogen, 73% of which was supplied by urea, varied from 43 to 55 with a mean of 52.

The four calves fed the urea diet were slaughtered at 4 months of age and the edible meat subjected to analyses. On the fresh basis the carcass meat contained 79.6% moisture, 18.2% protein, 1.37% fat and 0.85% mineral matter. In com-

parison with data reported by Moulton et al. ('22) for normal cattle of a similar age, these animals contained the same protein content, somewhat more water and less fat.

It is interesting that calves as young as 2 months of age are able to make fair gains in weight when urea constitutes about three-fourths of the dietary nitrogen. The added urea changed negative nitrogen balances to positive ones and permitted retention of 24 to 36% of the food nitrogen. That this nitrogen was stored primarily as body protein is suggested by the fact that the muscle tissue was normal in crude protein content.

Opportunity was taken to assay the organs and the edible meat for riboflavin content by the microbiological method of Snell and Strong ('39). The samples of the organs and meat of the calves fed the B vitamins contained no more riboflavin than samples from calves without the B vitamin supplements. The average riboflavin contents in micrograms per gram of fresh sample were: liver 36.5, kidney 22.9, heart 10.8, spleen 3.3 and muscle of entire carcass 1.8.

SUMMARY

Calves 2 months of age were unable to grow when they were fed a diet containing only 4.4% protein. When urea was added to the diet to give a calculated protein content of 16.2% ($N \times 6.25$) calves increased in body weight and height at fairly satisfactory rates. The calves fed the basal diet were in negative nitrogen balance while those receiving the added urea were in positive nitrogen balance, retaining 24 to 36% of the dietary nitrogen. The apparent digestibility of the dry matter and carbohydrates of the basal diet was 57 to 63%, while the dry matter and carbohydrates of the urea diet were 74 to 80% digested.

Feeding daily supplements of the B vitamins did not increase the rate of growth or the efficiency of nitrogen utilization.

The riboflavin content of the organs and edible meat was not increased by feeding supplements of the B vitamins.

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FAT METABOLISM AND GOITER [1]

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(Received for publication September 21, 1942)

In an earlier contribution from this laboratory (Remington, '38) evidence was presented in refutation of the theory that the metabolism of fat involves a drain on the thyroid mechanism which is conducive to hypertrophy and hyperplasia of the gland. In that work fat, both saturated and unsaturated, was added to a previously compounded low iodine diet which experience had shown to be uniformly productive of a significant degree of goiter, but which was otherwise adequate for fairly good growth and reproduction in rats. The highest level of fat employed supplied about 30% of the caloric intake, which is not abnormally high for some self-selected human diets. As evidenced by size, degree of edema, and iodine storage in the thyroid gland, there was no extra drain on this gland due to the added fat. However, the addition of 10% of fat to the previously compounded basal diet so decreased its supply of factors, presumably of the B complex, as to result in impaired growth and loss of hair. This rendered it impossible to determine the effect of higher amounts of fat, and also introduced the question of the effect of varying growth rates on iodine requirement.

Accordingly, several experiments were performed to devise a diet that would be more abundantly supplied with protective factors without lessening its goiter-producing property, and to so balance the protein and protective foods that the supply of each would be more nearly constant. This was accomplished

[1] Aided by a grant from the Committee on Scientific Research of the American Medical Association.

by the addition of 5% of dried brewers' yeast (Remington, '37), and by progressively increasing the proportion of wheat gluten, liver, and yeast as fat was introduced into the diet, the aim being to provide as nearly as possible a constant ratio between protective factors and energy value. The iodine content of the diets was not significantly different when calculated on a calorie basis. The values for fat, protein, and calories of ingredients were taken from published average analyses, and therefore are not rigidly accurate, but sufficiently so for our purpose. Then, in order to assure comparable utilization of the diets fed, food intakes were restricted to an amount which would produce considerably less than normal growth, and limited in the different groups in such a way as to produce practically uniform growth in all animals. In this way we were able to introduce as much as 30% of fat into the diet, which with that present in other ingredients accounted for 56% of the energy value.

TABLE 1

Effect on the thyroid gland of iodine deficient diets varying in fat content, when fed to rats at substantially iso-caloric levels.

DIET NUMBER	374	383	384	385	386
Composition					
Whole yellow corn (ground)	73.0	45.0	30.7	45.0	30.7
Wheat gluten	18.0	24.1	27.1	24.1	27.1
Dried brewers' yeast	5.0	6.6	7.4	6.6	7.4
Dried pig liver	2.0	2.3	2.8	2.3	2.8
Sodium chloride	1.0	1.0	1.0	1.0	1.0
Calcium carbonate	1.0	1.0	1.0	1.0	1.0
Linseed oil		20.0	30.0		
Hydrogenated vegetable oil [1]				20.0	30.0
Per cent of total calories as:					
Protein	18.8	15.5	14.5	15.5	14.5
Fat	9	46	56	46	56
Average weight gain in grams (8 weeks)	55	51	57	53	55
Thyroid glands					
Weight (mg. per 100 gm.)	16.0±1.1	19.6±0.9	20.6±1.1	16.6±0.7	16.1±0.5
Dry matter (per cent)	25.1	23.4	24.5	23.0	23.6

[1] Crisco.

EXPERIMENTAL FINDINGS

Each group consisted of nine young rats, uniformly divided as to sex and litter. The experimental period was 8 weeks. At the end of the experiment the rats were chloroformed and all thyroid glands removed for examination and chemical analysis. In appearance they were all hemorrhagic and enlarged. The statistical studies of Remington, Remington and Welch ('37) have shown that on rats of comparable size reared on a satisfactory colony ration adequately provided with iodine, the thyroid glands showed the following values: fresh weight 9.8 ± 0.5 mg. per 100 gm. body wt., dry matter 32.0%, iodine 0.2% dry basis. Compared with these values, the rats of the present series showed a degree of enlargement about double; the dry matter content was about 75%, i.e., there was marked edema. Iodine was determined on the pooled glands of each group. The values were rather uniform, and all less than 0.02% dry basis, i.e., less than 10% of normal. Differences in thyroid weight among groups were not statistically significant.

CONCLUSIONS

Since the iodine content of the glands, and the degree of enlargement and edema produced were within a critical range where variations in the drain on the thyroid mechanism, if such existed, would inevitably produce a considerable change in the state of the glands (Remington and Levine, '36), it seems assured that the function of the thyroid gland is not specifically related to the metabolism of fat, either saturated or unsaturated, at least at levels lower than that which might be expected to produce ketosis.

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